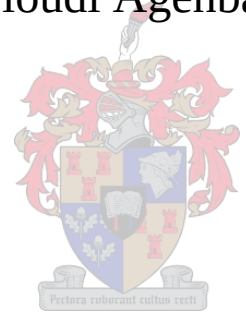
Molecular Genetic Analysis of Familial Breast Cancer in South Africa

By

Gloudi Agenbag



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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: _____ Date: _____

ABSTRACT

Breast cancer is a major cause of morbidity and mortality as it is the most common invasive cancer in women worldwide. The lifetime risk for South African women to develop breast cancer is one in 31. A family history of the disease is a well-established risk factor and germline mutations in the *BRCA1* (breast cancer one) and *BRCA2* (breast cancer two) tumour suppressor genes markedly increase the risk of developing breast cancer. A few hundred mutations spanning the entire coding sequences of both genes have already been reported. Numerous other breast cancer susceptibility loci have been identified, but results from association studies are discrepant. The checkpoint kinase gene, *CHEK2*, and specifically the *CHEK2**1100delC variant has, however, consistently been implicated as a candidate low-penetrance breast cancer allele. To date, few comprehensive molecular-genetic studies have been completed for the various South African breast cancer populations.

The aim of this study was to determine the *BRCA1* and *BRCA2* mutation spectrum and prevalence in two South African populations, namely Mixed Ancestry and Caucasian. The frequency of the *CHEK2**1100delC mutation was also investigated. The patient group comprised 101 unrelated patients (98 women and 3 men), presenting with invasive breast cancer. Patients with a moderate family history of breast cancer (n=48) were screened for the *CHEK2**1100delC allele and the coding sequences of the *BRCA1* (partly completed in a previous study) and *BRCA2* genes. Patients without a family history of the disease (n=53) were only screened for the *CHEK2**1100delC allele. Mutation detection was done using combined single-strand conformation polymorphism and heteroduplex analysis (SSCP/HA), followed by DNA sequencing of the identified variants. Due to its size (~5kb), exon 11 of *BRCA2* was sequenced directly after amplification, in seven overlapping fragments.

Three deleterious *BRCA1* mutations, 1623_1627delTTAAA, E881X and 5313delC have previously been identified in three patients from the study population. No additional pathogenic mutations have been detected in this gene during this study. Two deleterious *BRCA2* mutations, 6677_6678insTA and 8162delG, were identified in two and three patients respectively. Overall, *BRCA1* and *BRCA2* mutations have been identified in 17% of the Mixed Ancestry patients and in 15.8% of the Caucasian patients. Together *BRCA1* and *BRCA2* mutations account for 16.7% of breast cancer in the study population. In addition, a number of silent polymorphisms as well as variants of unknown functional significance, both known and novel, were identified.

The E881X variant, which has been reported as an Afrikaner founder mutation (Reeves *et al.* 2004), was identified in one patient of Mixed Ancestry, but none of the published European founder mutations have been detected in our patient group. This suggests a unique mutation spectrum for South African breast cancer patients. The prevalence of the *BRCA2* mutations, 8162delG and 6677_6678insTA, has to be elucidated within a larger study group. Haplotype analysis will reveal whether these patients have a common ancestor. Our findings do not suggest the presence of the *CHEK2* variant in South African breast cancer patients, but a larger study population has to be analysed to confirm this.

The results of this study are in agreement with those from other populations, indicating that less than 20% of breast cancers that occur in individuals with a moderate-risk for developing breast cancer are due to *BRCA1* and *BRCA2* mutations. By determining the contribution of *BRCA1* and *BRCA2* mutations to breast cancer in this group of patients, one can assess the appropriateness of predictive or diagnostic DNA testing in the clinical setting.

OPSOMMING

Borskanker is ’n belangrike oorsaak van morbiditeit en sterftes aangesien dit die mees algemene vorm van kanker in vroue wêreldwyd is. Die risiko vir ’n Suid-Afrikaanse vrou om borskanker te ontwikkel is een in 31. ’n Familiegeskiedenis van die siekte is ’n bevestigde risikofaktor, en kiemlyn-mutasies in die *BRCA1* (“breast cancer one”) en *BRCA2* (“breast cancer two”) gewasonderdrukker gene verhoog die risiko om borskanker te ontwikkel aansienlik. ’n Paar honderd mutasies in die koderende volgordes van beide gene is reeds gerapporteer. Talryke ander borskanker vatbaarheidslokusse is reeds geïdentifiseer, maar resultate van assosiasie-studies is teenstrydig. Die sel-sikluskontrolepunt geen, *CHEK2*, en meer spesifiek die *CHEK2**1100delC variant, is konsekwent aangedui as ’n moontlike lae-penetrasie borskanker-alleel. Tot op hede is min omvangryke molekulêre genetika studies gedoen oor borskanker in die onderskeie Suid-Afrikaanse bevolkingsgroepe.

Die doel van hierdie studie is om die *BRCA1* en *BRCA2* mutasiespektrum en voorkoms te bepaal in twee Suid-Afrikaanse bevolkingsgroepe, naamlik Gemengde Herkoms (Kleurling) en Kaukasiër. Die frekwensie van die *CHEK2**1100delC mutasie is ook vasgestel. Die pasiëntegroep het bestaan uit 101 onverwante individue (98 vroulik en 3 manlik) met borskanker. Pasiënte met ’n familiegeskiedenis van borskanker (n=48) is gesif vir die *CHEK2**1100delC alleel en vir die koderende volgordes van die *BRCA1* (gedeeltelik afgehandel in ’n vorige studie) en *BRCA2* gene. Pasiënte sonder ’n familiegeskiedenis van die siekte (n=53) is slegs gesif vir die *CHEK2**1100delC alleel. Mutasie-opsporing is gedoen met behulp van enkelstring konformasie polimorfisme en heterodupleks analise (SSCP/HA), gevolg deur DNS-volgordebepaling van die geïdentifiseerde variante. As gevolg van sy grootte (~5kb), is ekson 11 van *BRCA2* in sewe oorvleuelende fragmente geanaliseer deur middel van direkte volgordebepaling ná amplifikasie.

Drie patogeniese *BRCA1* mutasies, 1623_1627delTTAAA, E881X en 5313delC, is voorheen geïdentifiseer in drie pasiënte uit die studiegroep. Geen bykomende mutasies is opgespoor in die geen gedurende hierdie studie nie. Twee patogeniese *BRCA2* mutasies, 6677_6678insTA en 8162delG, is geïdentifiseer in twee en drie pasiënte onderskeidelik. In die geheel is *BRCA1* en *BRCA2* mutasies geïdentifiseer in 17% van die Gemengde Herkoms pasiënte en in 15.8% van die Kaukasiër pasiënte. Gesamentlik is *BRCA1* en *BRCA2* mutasies verantwoordelik vir 16.7% van borskanker in die

studiegroep. Daarbenewens is ’n aantal bekende en nuwe polimorfismes, asook variante waarvan die funksionele betekenis onbekend is, geïdentifiseer.

Die E881X variant, wat gerapporteer is as ’n Afrikaner stigtermutasie (Reeves *et al.* 2004), is geïdentifiseer in een pasiënt van Gemengde Herkoms, maar geen algemeen bekende Europese stigtermutasies is gevind in die pasiënt groep nie. Dit dui op ’n unieke mutasiespektrum vir Suid-Afrikaanse borskankerpasiënte. Die voorkoms van die *BRCA2* mutasies, 8162delG en 6677_6678insTA, behoort bepaal te word in ’n groter studiegroep. Haplotipe analise sal aan die lig bring of hierdie pasiënte ’n gemeenskaplike voorouer het. Die bevindings van hierdie studie toon nie die teenwoordigheid van die *CHEK2* variant in die Suid-Afrikaanse bevolking nie, maar ’n groter studiegroep moet ondersoek word om dit te bevestig.

Die resultate van hierdie studie is in ooreenstemming met studies in ander bevolkings, wat daarop dui dat minder as 20% van borskankers wat voorkom in individue met ’n matige risiko vir borskanker, as gevolg van *BRCA1* en *BRCA2* mutasies is. Met die vasstelling van die bydrae wat *BRCA1* en *BRCA2* mutasies lewer tot borskanker in die pasiëntgroep, kan die toepaslikheid van DNS-toetsing in ’n kliniese omgewing vasgestel word.

List of Abbreviations and Symbols

%	Percentage
®	Registered trademark
°C	Degrees Celsius
A	Adenine (in DNA sequence)
Acetic acid	CH_3COOH
Acrylamide	C_3H_5NO
AC±T	Adriamycin, Cytoxan and Taxol or Taxotene (chemotherapy)
ADH	Atypical ductal hyperplasia
AF-2	Activation function domain of ER-α
AI	Aromatase inhibitors
Ala	Alanine (amino acid)
ALH	Atypical lobular hyperplasia
Alu	Short interspersed nuclear element, characterised by the *Alu*I restriction enzyme
APS	Ammonium persulphate
AR	Androgen receptor
Arg	Arginine (amino acid)
Asn	Asparagine (amino acid)
ASO	Allele specific oligonucleotides
Asp	Aspartic acid (amino acid)
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and RAD3 related
Aurora-A	Aurora kinase A (Aurora2/STK15/BTAK)
BACH1	BRCA1-associated carboxyl-terminal helicase
BAP1	BRCA1-associated protein
BARD1	BRCA1-associated RING domain
BASC	BRCA1-associated genome surveillance complex
BC	Breast cancer
BIC	Breast Cancer Information Core
Bisacrylamide	N,N'-methylene-bis-acrylamide: $C_7H_{10}O_2N_2$
BLM	Bloom syndrome (DNA helicase RecQ protein-like-3, RECQL3)

Boric acid	H_3BO_3
bp	Base pair
BRAF35	BRCA2-associated factor
BRC	BRCA2 repeat motif
Brca1	*BRCA1* murine homolog
BRCA1	Breast cancer susceptibility gene 1
Brca2	*BRCA2* murine homolog
BRCA2	Breast cancer susceptibility gene 2
BRCA3	Breast cancer susceptibility gene 3
BRCA2DBD	BRCA2 DNA/DSS1-binding domain
BRCT	BRCA1 carboxyl-terminal
BRIP1	BACH1-BRCA1-associated C-terminal helicase-1
Bromophenol blue	3',3'',5',5''-tetrabromophenolsulfonephthalein: $C_{19}H_{10}Br_4O_5S$
BTAK	Breast tumour amplified kinase (Aurora-A/STK15)
C	Cytosine (in DNA sequence)
cAMP	3'5'-cyclic adenosine monophosphate
Canis familiaris	Dog
CANSA	Cancer Association of South Africa
CCD	Charge-coupled device
Cdc2	Cell cycle division 2
Cdc25A	Cell cycle division 25A
Cdc25C	Cell cycle division 25C
CDK2	Cyclin-dependent kinase 2
cDNA	Complementary deoxyribonucleic acid
CDS1	CDP-deacylglycerol synthase 1
CDP	Phospatidate cytidylyltransferase 1
CH_3COOH	Acetic acid
CHEK2/*CHK2*	Checkpoint kinase 2 gene
CMF	Combination of Cytoxan, Methotrexate and 5-Fluorouracil (chemotherapy)
COMT	Catechol-*O*-methyltransferase
CSGE	Conformation sensitive gel electrophoresis
CtBP	C-terminal-binding protein
CtIP	CtBP-interacting protein
CYP	Cytochrome P450 superfamily

D	Aspartic acid (amino acid)
dATP	2’-Deoxyadenosine-5’-triphosphate
DCIS	Ductal carcinoma *in situ*
dCTP	2’-Deoxycytidine-5’-triphosphate
ddNTP	Dideoxynucleotide triphosphate
del	Deletion
DGGE	Denaturing gradient gel electrophoresis
dGTP	2’-Deoxyguanosine-5’-triphosphate
dH_2O	Distilled water
dHPLC	Denaturing high-performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPD2	Dopamine receptor D2
DS	DNA sequencing
DSB	Double strand break
dsDNA	Double-stranded DNA
DSS1	Deleted in split-hand/split-foot 1 region
dTTP	2’-Deoxythumidine-5’-triphosphate
DZ	Dizygotic
E	Glutamic acid (amino acid)
EDTA	Ethylenediamine tetra-acetic acid: $C_{10}H_{16}N_2O_8$
e.g.	Exempli gratia (Latin abbreviation for “for example”
ER	Estrogen receptor
ERD	Estrogen receptor down-regulators
et al.	Et alia (Latin abbreviation for “and others”)
EtBr	Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide): $C_{21}H_{20}BrN_3$
EtOH	Ethanol: CH_3CH_2OH
F	Phenylalanine (amino acid)
FANCD1	Fanconi anaemia complementation group 1
FCCM	Fluorescent chemical cleavage of mismatch
FH	Familial hypercholesterolemia
FHA	Fork head associated
FNAB	Fine needle aspiration biopsy

Formaldehyde	HCHO
Formamide	$HCONH_2$
G	Glycine (amino acid)
g	Gram
G	Guanine (in DNA sequence)
G1-phase	Growth phase 1 of cell cycle
G2-phase	Growth phase 2 of cell cycle
GADD45	Growth-arrest- and DNA damage-inducible 45
gDNA	Genomic deoxyribonucleic acid
Gln	Glutamine (amino acid)
Glu	Glutamic acid (amino acid)
Gly	Glycine (amino acid)
Glycerol	$C_3H_5(OH)_3$
GSTM1	Glutathione *S*-transferase-μ1
GSTT1	Glutathione *S*-transferase-θ 1
H	Histidine (amino acid)
H2AX	histone species
HA	Heteroduplex analysis
HBCC	Hereditary breast and colorectal cancer
HCHO	Formaldehyde
He	Heterozygous
HER-2	HER-2 protein, derived from human EGFR (epidermal growth factor receptor) 2, also known as c-erbB-2 or *neu*
His	Histidine (amino acid)
Ho	Homozygous
HR	Homologous recombination
HRAS1	Harvey rat sarcoma viral oncogene homolog 1
HRT	Hormone replacement therapy
HWE	Hardy-Weinberg equilibrium
I	Isoleucine (amino acid)
ICAM	Intracellular adhesion molecule
IDC NOS	Ductal carcinoma not otherwise specified
i.e.	Id est (Latin abbreviation for “that is”)
ILC	Invasive lobular carcinomas

Ile	Isoleucine (amino acid)
Inc	Incorporated
ins	Insertion
in utero	Latin abbreviation for “in the uterus”
in vitro	Latin abbreviation for “in a test tube”
IR	Ionizing radiation
IVS	Intervening sequence
K	Lysine (amino acid)
kb	Kilo base pair
kD	Kilo Dalton
L	Leucine (amino acid)
LCIS	Lobular carcinoma *in situ*
LDLR	Low-density lipoprotein receptor
Leu	Leucine (amino acid)
LH	Luteinizing hormone-releasing
LKB1	Serine/threonine protein kinase 11 (STK11)
LOH	Loss of heterozygosity
Lys	Lysine (amino acid)
M	Methionine (amino acid)
M	Molar (moles per litre)
M	Pathogenic mutation
melt-MADGE	Melt-point analysis by microplate-array diagonal-gel electrophoresis
Met	Methionine (amino acid)
mg/ml	Milligrams per millilitre
MIM	Mendelian Inheritance in Man
ml	Millilitre
mm	Millimetre
mM	Millimolar
M-phase	Mitotic phase of cell cycle
MPLA	Multiplex ligation-dependent probe amplification
MRE11	Mitotic recombination 11
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSH1	Bacterial *Muts* homolog 1

MSH2	Bacterial *Muts* homolog 2
MSH6	Bacterial *Muts* homolog 6
MTHFR	Methylenetetrahydrofolate reductase
Mus musculus	House mouse
*Mva*I	Restriction endonuclease isolated from *Micrococcus varians* RFL19, with recognition site 5'-CC↓WGG-3'
MYC	Myelocytomatosis viral oncogene homolog
MZ	Monozygotic
N	Asparagine (amino acid)
n	Sample size
NAT1	*N*-acetyltransferase 1
NAT2	*N*-acetyltransferase 2
NBR1	Next to *BRCA1* one gene
NBR2	Next to *BRCA1* two gene
Nbs1	Nijmegen breakage syndrome 1
NCBI	National Center for Biotechnology Information
ng	Nanogram
ng/µl	Nanograms per microlitre
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
NOS	Not otherwise specified
NuMA	Nuclear mitotic apparatus
OB1	Oligonucleotide/oligosaccharide-binding 1
OB2	Oligonucleotide/oligosaccharide-binding 2
OB3	Oligonucleotide/oligosaccharide-binding 3
OCCR	Ovarian cancer cluster region
OR	Odds ratio
p	Chromosome short arm
p	Pagination (page number)
P	Polymorphism
P	Probability
P	Proline (amino acid)
P/CAF	p300/CBP-associated factor
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PM5	Polymorphism 5
PM6	Polymorphism 6
PM7	Polymorphism 7
pmol/µl	Picomolar per microlitre
PR	Progesterone receptor
Pro	Proline (amino acid)
pS2	Presenilin 2
PTEN	Phosphatase and tensin homolog
PTT	Protein truncation test
q	Chromosome long arm
Q	Glutamine (amino acid)
R	Arginine (amion acid)
RAD51	Eukaryotic homolog of bacterial RecA
RAD53	*Saccharomyces cerevisiae* homolog of human *CHEK2*
RB	Retinoblastoma
rcf	relative centrifugal force
RFLP	Restriction fragment length polymorphism
RING	Zinc-chelating domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Serine (amino acid)
SDS	Sodium dodecyl sulphate: $C_{12}H_{25}NaSO_4$
Ser	Serine (amino acid)
SERM	Selective estrogen receptor modulators
Silver nitrate	$AgNO_3$
S-phase	DNA synthesis phase of cell cycle
SSA	Single-strand annealing
SSCP	Single strand conformation polymorphism
ssDNA	Single-stranded DNA
STK11	Serine/threonine protein kinase 11 (LKB1)
STK15	Serine/threonine protein kinase 15 (Aurora-A/BTAK)
T	Threonine (amino acid)

T	Thymine (in DNA sequence)
Taq	DNA polymerase (EC 2.7.7.7) isolated from *Thermus aquaticus*
TBE	Tris borate-EDTA buffer (5X TBE: 0.089M Tris; 0.089M boric acid; 0.002M EDTA)
TDLU	Terminal duct lobular units
TEMED	N,N,N',N'-tetrametylethylenediamine
Temp	Temperature
TFPGA	Tools for Population Genetic Analysis
TGGE	Thermal gradient gel electrophoresis
Thr	Threonine (amino acid)
TP53	Tumour protein 53
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$
Trp	Tryptophan (amino acid)
™	Trademark
U	Units
UK	United Kingdom
Urea	CH_4N_2O
US	United States
UTR	Untranslated region
UV	Ultraviolet
UV	Unclassified variant
V	Valine (amino acid)
V	Volts
v/v	Volume per volume
VDR	Vitamin D receptor
Versus	Latin abbreviation for "against"
VNTR	Variable number of tandem repeat
W	Adenine or Thymine (in DNA sequence)
w/v	Weight per volume
WT	Wild type
X	Stop codon (UAA, UAG or UGA)
XRCC1	X-ray repair cross-complementation group 1
XRCC2	X-ray repair cross-complementation group 2
XRCC3	X-ray repair cross-complementation group 3

Xylene cyanole	Xylene cyanole FF: $C_{25}H_{27}N_2NaO_6S_2$
ZBRK1	Zinc finger and *BRCA1* interacting protein with a KRAB domain 1
α	Alpha
β	Beta
μ	Mu
μg/ml	Micrograms per millilitre
μM	Micromolar
ψ*BRCA*1	*BRCA1* pseudogene

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TABLE OF CONTENTS

1. INTRODUCTION

Mammary carcinoma (breast cancer MIM #114480) refers to cancer of the breast epithelial tissue, a histopathologically, etiologically and genetically heterogeneous disease. Currently it is the most common invasive cancer in women worldwide, with incidence rates the highest in industrialized nations such as the United States, Australia and countries in Western Europe (Ghafoor *et al.* 2003). Although the mortality rate has decreased during the 20th century, incidence has increased (Althuis *et al.* 2005). This trend can only partly be accounted for by the increased use of early diagnostic and screening techniques. The risk of a woman developing breast cancer during her lifetime is one in seven among women from the United States (Jemal *et al.* 2005), one in nine among British women (UK National Statistics Online www.statistics.gov.uk) and one in 66 among Japananese women (Japan National Cancer Centre www.ncc.go.jp). The lifetime risk for South African women to develop breast cancer is one in 31 (CANSA statistics www.cansa.org.za). Two-thirds of breast cancers are detected in postmenopausal women.

Breast cancer remains a major cause of morbidity and mortality worldwide and despite intensive research efforts, consensus agreement on management has proved difficult. This could partly be attributed to the complexity of the disease, which has widely differing biological backgrounds. Understanding the molecular basis of malignancy will allow more rational surveillance, prevention and treatment in future.

A family history of breast cancer has been a well-established risk factor for the disease for more than five decades (Gardner and Stephens 1950). Many impressive pedigrees supporting the existence of these dominant highly penetrant breast and/or ovarian cancer genes have been published, but the first convincing localization of a breast cancer gene, *BRCA1* (MIM #113705), was only achieved in 1990 by linkage analysis (Hall *et al.* 1990). A second gene, *BRCA2* (MIM #600185), was mapped in 1994 (Wooster *et al.* 1994). Although mutations in the *BRCA1* and *BRCA2* genes may explain the majority of familial breast cancer (refer to chapter two for a detailed review of the literature), familial breast cancer has been repeatedly shown to constitute only 5-10% of all breast cancer cases.

Germline mutations in the *BRCA1* and *BRCA2* tumour suppressor genes markedly increase the risk of developing breast and ovarian cancer. They are examples of Mendelian inheritance of susceptibility to a common, genetically complex, adult-on-set disease. One defective copy of *BRCA1* or *BRCA2* in the germline may be enough to cause cancer predisposition, but the wild-type allele is often inactivated through loss of heterozygosity (LOH) with retention of the mutant allele (Smith *et al.* 1992). The exact magnitude of this risk, that is, the penetrance of mutations in these genes, remains controversial. The *CHEK2* gene (MIM #604373), which is a cell cycle checkpoint kinase, has recently been shown (Meijers-Heijboer and The *CHEK2*-Breast Cancer Consortium 2002) to be a plausible candidate low-penetrance breast cancer gene. Various other breast cancer susceptibility loci have been identified, including putative *BRCA3* loci; unfortunately none of these have been clearly elucidated.

BRCA1 and *BRCA2* are both relatively large genes and a few hundred mutations spanning the entire coding sequences of both have already been reported (Breast Cancer Information Core database http://research.nhgri.nih.gov/bic). Mutation frequencies vary among different ethnic and geographically distinct populations. Almost all studies performed to date have defined breast cancer risks and related genetic factors in women of European descent. Few *BRCA1* and *BRCA2* studies have been done in any of the African populations or in the African American population. Even less comprehensive molecular-genetic studies have been completed for the various South African populations. To be able to implement predictive genetic testing for *BRCA1* and *BRCA2* mutations successfully in South Africa, the genes have to be characterised in South African breast cancer families. The mutation spectrum has to be determined and possible founder mutations identified. The molecular implications of new variants have to be elucidated in order to distinguish benign polymorphisms from pathogenic mutations. We are boldly moving into the age of genetics, by joining forces with patients, health care professionals, genetic counsellors and biotechnology companies, to define the settings in which genetic testing will become an indispensable tool used to improve clinical outcomes.

Chapter two provides a detailed look at breast cancer as a disease, both clinically and molecularly. The functions of the *BRCA1*, *BRCA2* and the *CHEK2* gene in the normal cell cycle are discussed as well as the role these genes play in malignant progression. Available literature is presented to assess the relationship these genes have to hereditary breast cancer. In chapter three the materials and methods used to conduct this study are presented. Results are reported in chapter four, followed by a discussion

of the results in chapter five, which concludes this study with a short discussion of the relevance of this particular study for South African breast cancer patients.

2. LITERATURE REVIEW

2.1 BREAST CANCER INCIDENCE AND EPIDEMIOLOGY IN SOUTH AFRICA

The source of information on cancer incidence and mortality in South Africa is the National Cancer Registry, which collects information from pathology laboratories on cancers histologically diagnosed (Vorobiof *et al.* 2001). These statistics, which are reported on the CANSA website (www.cansa.org.za), are an underestimate of the true incidence of cancer, since not all individuals have their cancer clinically diagnosed. In South Africa a total of 4789 new cases of female breast cancer were reported in 1997 (16.4% of all cancers reported). Incidence rates vary enormously between South Africa's ethnic groups, a trend that is at present not completely understood. The lifetime risk (null to 74 years) for women is one in 31 overall, but varies from one in 57 among African, one in 16 among Caucasian and Mixed Ancestry, to one in 13 among Asian women. Previously, incidence was calculated separately for Caucasian and Mixed Ancestry women. The lifetime risk was one in 13 among Caucasian and one in 63 among women from Mixed Ancestry. For Caucasian and Mixed Ancestry men the lifetime risk is one in 667. Age standardised rates (expressed in new cases per 100 000 people per year) for Caucasian South Africa women from 1993 to 1995 (70.2 per 100 000) was comparable to rates from developed countries, such as the United Kingdom (114 per 100 000) (UK National Statistics Online www.statistics.gov.uk) and the United States (135 per 100 0000) (Jemal *et al.* 2005). In 1997 the combined age standardized rate for Caucasian and Mixed Ancestry women was 55.49 per 100 000, but the average rate for all racial groups was only 29 women per 100 000 and 1.1 men per 100 000.

2.2 NORMAL STRUCTURE OF THE BREAST

The breast is a collection of glands, fibrous connective tissue and adipose tissue that lies between the skin and the chest wall. The glands are called lobules, and many lobules make up a lobe. There are about 15 to 20 lobes in each breast. Milk is transported to the nipple from the glands by way of tubes called the ducts. A system of 15 to 20 ducts, converging at the nipple, branch out into the fatty mass of the breast and end in thousands of lobules (refer to Figure 2.1). The lobules are small secretory units, called the terminal duct lobular units (TDLU) (Russo and Russo 2004). The TDLU are highly folded

globular aggregates of tubules in a cellular stroma, which is composed of specialized and functionally active mesenchymal cells. The stromal cells secrete, sequester or activate cytokines and growth factors and secrete matrix components, all of which influence growth and differentiation of the epithelial compartment. Each tubule is lined by an inner layer of cuboidal epithelial cells surrounded by a layer of basal or myoepithelial cells, which are a network of specialized cells with both epithelial and mesenchymal characteristics. The myoepithelial cells secrete the basement membrane of the secretory units and have an influence on development and function of the TDLU. The TDLU can enlarge and unfold under hormonal stimulation (e.g. lactation) or in neoplasia, giving rise to larger diameter spaces. The breast epithelial and stromal cells are responsive to female hormones, particularly estrogen, progesterone and prolactin, and express the corresponding receptors. Androgens cause atrophy of these cells (Reviewed in Stamp 2002).

There are also blood vessels and lymph vessels in the breast. Most of the lymph nodes that drain the breast are under the arm in what is called the axilla.

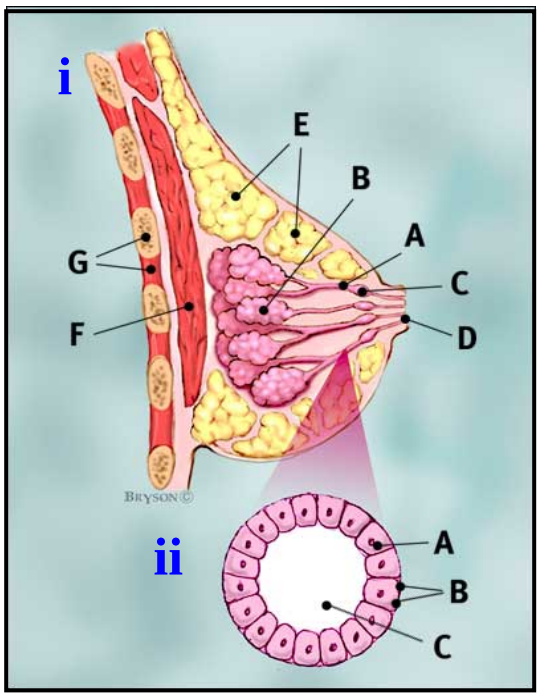

Figure 2.1 i) A schematic profile of the breast: A – ducts, B – lobules, C - dilated section of a duct as at times of lactation, D – nipple, E – adipose tissue, F - pectoralis major muscle, G - chest wall and rib cage. **ii) Enlargement of a duct:** A - normal duct cells, B - basement membrane, C - lumen. Reprinted with permission from BreastCancer.org (www.breastcancer.org) and MK Bryson.

2.3 TYPES OF BREAST CANCER

Breast cancers may be biopsied for histological examination using a fine gauge needle to extract a cell sample (fine needle aspiration biopsy, FNAB) or by extracting a tissue core. Alternatively a surgical biopsy is undertaken under anaesthesia, removing part of or the entire lesion. Axillary lymph nodes are sampled to assess for the presence of metastasis. The term non-invasive or *in situ* carcinoma is used when the cancer cells are confined to the ducts or lobules where it initially developed. Invasive or infiltrating carcinoma means that the cancer cells have invaded surrounding adipose and connective tissues of the breast. Stage 0 is used to describe non-invasive breast cancer, with stages I, II and IIIA describing invasive breast cancer according to the size of the tumour, with (stage II and IIIA) or without (stage I) the involvement of the lymph nodes. Stage IIIB describes invasive breast cancer in which a tumour of any size has spread to the breast epidermis, the chest wall or to the internal mammary lymph nodes. Stage IV is the most advanced form of breast cancer with definite metastasis to other organs (Harris 1991).

Breast tumours include several histopathological subtypes. Within many of these subtypes there exists a spectrum of appearances. Tumours could be composed of relatively small, monotonous cells with a low rate of cell proliferation showing some features of normal breast tissue organization. These types of tumours are associated with a relatively benign prognosis. Conversely, tumours could be composed of pleomorphic cells with little evidence of normal tissue architecture and these cancers are associated with relatively poor outcome. Pathologists use different grading systems to qualify and quantify the neoplasm. The Van Nuys prognostic index uses lesion size, nuclear grade and the presence or absence of necrosis as scoring parameters (Silverstein *et al*. 1995). Another grading system is the modified Bloom and Richardson system (Elston and Ellis 1991), which assesses the architecture by estimating a) the proportion of the tumour in which the epithelial cells organize themselves into tubular structures, b) the cytology by the size and regularity of the nucleus (pleomorphism), and c) the biological behaviour by the number of mitotic figures per unit area.

A simplified discussion of the main histological subtypes follows. Tumours could consist entirely of only one type, or could be a combination of any of these (Reviewed in Stamp 2002).

2.3.1 *In situ* carcinoma

In situ carcinomas arise within the TDLU. They may spread to adjacent TDLU, but usually do not bridge the basement membrane. If the cells do acquire the ability to bridge the basement membrane, they become invasive. *In situ* carcinomas can assume a variety of patterns, including **cribriform** (aggregates of epithelial cells arranged around well-defined lumina), **micropapillary** (peripherally organized projections of epithelial cells towards a common central lumen), **solid** (with few organized lumina, but rather wall-to-wall cell growth) and **comedo** (similar to the solid pattern but with areas of necrosis, which indicates that it is a fast growing tumour) (Jaffer and Bleiweiss 2002).

Lobular carcinoma *in situ* (LCIS) develops inside the lobules and tends to have a monotonous appearance compared to a lot of structural variation seen in ductal carcinoma *in situ* (DCIS), which is confined to the ducts. Compared to DCIS, LCIS cells tend to be of uniform size with round to oval nuclei, less pleomorphism and usually a lower mitotic index. DCIS is the most common kind of non-invasive breast cancer. Atypical ductal hyperplasia (ADH) is a small lesion, usually involving part of the TDLU, where the proliferation does not display all of the features of DCIS. Atypical lobular hyperplasia (ALH) refers to cells that are cytologically similar to those of LCIS, but less extensively involve the TDLU and small ducts. ALH and LCIS are usually grouped together under the term "lobular neoplasia" (Simpson *et al.* 2003).

2.3.2 Invasive carcinoma

2.3.2.1 Invasive ductal carcinoma

Invasive or infiltrating ductal carcinoma not otherwise specified (IDC NOS) are composed of epithelial cells arranged in groups which show varying degrees of organization into glandular and tubular elements. These tumours do not have special features that are characteristic of the other types encountered, hence the designation "not otherwise specified" (NOS) (Fisher 1977). Tumours initially begin in the ducts, but soon spread into the surrounding tissue. This is the most common type of breast cancer and accounts for approximately 70% of all breast cancers (Rahman and Stratton 1998). There are some variants of IDC which have a distinctive appearance. **Tubular carcinoma** is composed of well-formed tubular structures whereas **infiltrating cribriform carcinoma** has tubular elements which

are complex and have small regular lumina distributed within them. In **mucinous carcinomas** (also called colloid or gelatinous carcinoma), the bulk of the tumour is composed of gelatinous secreted mucin which contains dispersed aggregates of neoplastic cells (Fisher 1977). **Medullary carcinomas** are well circumscribed and are composed of large syncytial aggregates of very pleomorphic tumour cells with an intervening stroma densely infiltrated by lymphocytes and plasma cells (Fisher 1977). **Secretory carcinoma** repeats the lactational phenotype and has large vacuolated cells lining spaces which contain the secrete.

2.3.2.2 Invasive lobular carcinoma

Invasive lobular carcinomas (ILC) originate in the lobules. Like IDC NOS, ILC is poorly cohesive and do not form large, well-defined, glandular elements. Cells tend to invade independently. About 10% of all breast cancers are of this type (Cocquyt and Van Belle 2005). There are a number of variant patterns, including the **solid** pattern, **pleomorphic** and **signet ring** variants (Weidner and Semple 1992). Better differentiated variants include the **alveolar** type, in which the cells form loose aggregates and the **tubulo-alveolar** type, where one will find poorly formed tubules.

2.3.2.3 Inflammatory breast cancer

Although this type of cancer is not common (about 1% of all breast cancers) it is an aggressive type of breast cancer that is considered stage IIIB. Inflammatory carcinoma is in fact a distinct clinicopathological subset of IDC (Felix 1999). The most distinguishing feature is the appearance of inflamed breasts with dimples and/or thick ridges caused by cells blocking lymph vessels in the breast epidermis. This type of cancer could be mistaken for an infection.

2.3.2.4 Paget's disease of the nipple

This is another rare form of breast cancer (also only occurring in 1% of all cases) which begins in the ducts and spreads to the skin of the nipple and areola. This type of cancer may be associated with *in situ* or invasive carcinoma. It could be caused by the spread of tumour cells from the ducts or lobules, but it may also be that the actual epithelial cells of the nipple spontaneously become cancerous.

Approximately 30-40% of women with Paget's disease have metastasis at the time of surgery (Felix 1999).

2.3.2.5 Other rare variants of breast carcinoma

A wide variety of uncommon differentiation patterns may be encountered in breast cancers. They include extreme variants of morphology or metaplastic differentiation such as **signet ring cell** carcinoma, **adenosquamous** and **squamous cell** carcinoma, **neuroendocrine** carcinoma, **apocrine carcinoma** and **clear cell** carcinoma. Metaplastic carcinomas can appear to undergo differentiation to distinct mesenchymal lineages, such as cartilage, osteoblasts or fibrosarcoma. Myoepithelial tumours display characteristics of both epithelial and mesenchymal cells, even to the extent of fully differentiating into smooth muscle cells during involution of the lobules. Another group of uncommon tumours which also seems to be composed of myoepithelial and epithelial components include **tubular adenomyoepithelioma**, **clear cell adenomyoepithelioma**, and **adenomyoepithela** with apocrine adenosis. **Fibroadenomas** represent a common hormone-dependent overgrowth of the TDLU, which rarely undergo malignant progression (Fisher 1977). **Phyllodes tumours** develop in the connective tissue and are stromal expansions which project into the epithelial compartment. These are true neoplasms which do not respond to hormonal therapy.

2.4 THE GENETICS OF BREAST CANCER

Breast cancer has been subdivided into two broad types, early-onset and late-onset, a division that is based on an inflection in the age-specific incidence curve around the age of 50 years (Miki *et al*. 1994). Alternatively, the disease can be classified as pre-menopausal or post-menopausal. The incidence in Western countries rises steeply with age until the age of about 50, and much more slowly thereafter. The conventional explanation for this is that breast mitotic activity is stimulated by estrogens and slows down at menopause (Easton 2000). Hereditary breast cancer is characterized by early age at onset (an average of five to 15 years earlier than sporadic cases), bilaterality (or multifocal tumours), vertical transmission through both maternal and paternal lines and familial association with tumours of other organs, particularly the ovary and prostate gland. Most breast cancers that are due to a genetic mutation occur before the age of 65. A woman with a strong family history of breast cancer of early-

onset who is still unaffected at 65 years, has probably not inherited the genetic mutation (McPherson *et al*. 2000). Familial breast cancer accounts for only a minority of all cases (5-10%).

Heterozygous germline mutations in the *BRCA1* tumour suppressor gene predispose one to early onset breast cancer as well as ovarian cancer. *BRCA2* mutation carriers are also predisposed to breast cancer, but generally have a later age of onset (Ford *et al*. 1998). *BRCA2* mutations are more strongly associated with male breast cancer than *BRCA1* mutations. It has been proposed that heterozygosity for *BRCA* mutations carry a phenotype that contributes to tumorigenic progression, instead of the loss of the wild type allele being stochastic. Heterozygosity for a *BRCA2* mutation has been shown to have a distinct phenotype, characterized by a reduced growth rate, increased cell death and heightened sensitivity to specific DNA damaging agents and reduced RAD51 (a eukaryotic homolog of bacterial RecA) focus formation after irradiation (Warren *et al*. 2003). Thus in certain cell types, genome instability might be driven directly by heterozygosity for *BRCA2* mutations and the same could be true for *BRCA1* mutations. Mice, heterozygous for *Brca1* or *Brca2* pathogenic mutations develop normally and do not appear to be more susceptible to mammary tumours than are mice with wild type *Brca1* and *Brca2*, while similar to humans, homozygous pathogenic mutations are embryonically lethal (Ludwig *et al.* 1997).

The role of the *BRCA1* and *BRCA2* genes in sporadic breast cancer remains unclear, since somatic mutations in either gene are uncommon (Gayther *et al.* 1998). Decreased levels of the *BRCA1* transcript have, however, been observed in several sporadic breast tumours suggesting that loss of regulation and mutations in noncoding regions may be mechanisms by which BRCA1 plays a role in the development of sporadic tumours (Thompson *et al.* 1995). Allele inactivation by promoter hypermethylation, which is an epigenetic effect, has been reported to occur at the *BRCA1* locus in somatic breast cancer (Dobrovic and Simpfendorfer 1997), but not at the *BRCA2* locus, even in those tumours with loss of heterozygosity (LOH) at this site (Collins *et al.* 1995; Collins *et al.*1997). This observation suggests that the mechanism of transcriptional repression is unlikely to explain the absence of somatic *BRCA1* and *BRCA2* mutations in sporadic cancers. Current pathologic, cytogenetic and gene expression data suggest that there are biological differences between *BRCA1*-related, *BRCA2*-related, and sporadic cancers. A clear understanding of these differences (and similarities) has not been reached yet.

Recent reports indicate that the proportion of breast cancer families attributable to the *BRCA1* and *BRCA2* genes may be smaller than initially thought (refer to paragraph 2.11.5 for a detailed discussion of the literature) and identification of additional breast cancer susceptibility genes is essential to be able to fully understand, prevent and treat the disease. Other candidate loci and specifically the *CHEK2* gene are discussed in paragraph 2.13. Other conditions associated with an autosomal dominant inheritance of breast cancer risk, but not *BRCA1* or *BRCA2* specifically, includes Li-Fraumeni syndrome (aberrant p53 expression), Cowden syndrome (most often due to mutant *PTEN*), Ataxia Telangiectasia (mutated copies of *ATM*) and Peutz-Jeghers syndrome (linked to *STK11/LKB1*).

2.5 MALE BREAST CANCER

Carcinoma of the male breast is a relatively rare disease that accounts for less than 1% of all cases of cancers in men and less than 1% of all diagnosed breast cancers (Sasco *et al.* 1993). In contrast to the increasing incidence of breast cancer in women, the incidence of breast cancer in men has remained stable over the past four decades. The median age at diagnosis spans ages 60-65 years, but the disease has been reported in males ranging from their mid-20s to their early 90s (Ravandi-Kashani and Hayes 1998). Incidence increases exponentially with age.

Carcinoma of the male breast has many similarities to breast cancer in women, but the disease has different genetic and pathologic features. Almost all of the histological subtypes of breast cancer that have been described in women have also been reported in men (Giordano *et al.* 2002). Approximately 90% of all breast tumours in men are invasive carcinomas. Although lobular cancer is more common in women, the vast majority of breast cancers in men are invasive ductal cancers. Carcinomas of the male breast have a higher rate of hormone receptor positivity (estrogen and progesterone) than do carcinoma of the female breast. As in women, axillary lymph node status, tumour size, histological grade and hormone receptor status have been shown to be significant prognostic factors in men with breast cancer (Giordano *et al.* 2002) and clinical outcome for men with breast cancer is similar to that for women. Bilateral disease is rare.

Many of the risk factors for breast cancer in men involve abnormalities in estrogen and androgen balance, which indicate that breast cancer in men, as in women, may be hormonally driven (Giordano *et al.* 2002). Other possible risk factors that relate to hormonal levels include obesity, which causes

increased peripheral aromatization of estrogens, and cirrhosis, which in turn results in a hyper estrogenic state. Other risk factors for breast cancer are testicular abnormalities, infertility, Klinefelter syndrome (47,XXY), benign breast conditions, radiation exposure, increasing age (Casagrande *et al.* 1988; Thomas *et al.* 1992; Sasco *et al.* 1993).

2.6 ENVIRONMENTAL FACTORS

Environmental risk factors have been intensively studied by epidemiologists. Nongenetic factors may significantly influence the penetrance of mutations. Identifying these nongenetic or environmental influences on penetrance suggests new directions for studies of *BRCA1*- and *BRCA2*-associated carcinogenesis.

One of the most important risk factors for breast cancer is age. For women the median age of onset is between 40 and 50 years (Easton *et al.* 1994). The incidence of breast cancer increases with age, doubling about every 10 years until menopause, when the rate of increase slows dramatically (McPherson *et al.* 2000). Geographic distribution appears to play another important role. The incidence and the mortality rate vary between different ethnically and geographically distinct populations by at least fourfold with the lowest incidence among Asians and the highest among North Americans, although the difference between Eastern and Western countries is diminishing. Studies on people who migrated from Japan to Hawaii showed that the rates of breast cancer in the migrants assumed the rate in the host country within one or two generations, indicating that environmental factors are of just as great importance as genetic factors (McPherson *et al.* 2000). Several studies have shown a birth cohort effect, whereby *BRCA1* and *BRCA2* mutation carriers born after 1930 or 1940 have a higher lifetime risk than women born before this time period (Antoniou *et al.* 2003; King *et al.* 2003).

The predominance of data from epidemiologic studies indicates that, aside from a family history of breast cancer and age, most breast cancer risk factors are related to reproductive events (Clavel-Chapelon and the E3N-EPIC Group 2002). Early menarche and late age at menopause have been shown to be risk factors. Nulliparity or late age at first birth (and also a late age at birth of a second child) increase the lifetime risk of breast cancer. Breast feeding reduces the risk of breast cancer. Lifestyle choices relating to these reproductive factors also play a role, for example the use of oral

contraceptives and hormone replacement therapy (HRT) (Li *et al.* 2003). Experimental data strongly suggest that estrogen, which can be linked to all above mentioned factors, have a role in the development and growth of breast tumours. Although the exact mechanisms remain to be fully elucidated, the alkylation of cellular molecules and the generation of active radicals, together with the potential genotoxicity of estrogen and some of its metabolites (e.g., the catechol estrogens), have been implicated (reviewed in Clemons and Goss 2001). Reactive oxygen species (ROS), generated in normal cellular respiration and during metabolism of xenobiotics, cause damage to membranes, mitochondria, and macromolecules including DNA and thus are plausible agents in the development of cancer. Estradiol is metabolized to ROS through redox cycling of the catechol estrogens and the semi-quinone radicals generated have been shown to result in DNA damage and upregulation of endogenous antioxidants in animal models (Cavalieri *et al.*2000).

Many epidemiological studies have investigated the relationship between breast cancer and the consumption of alcohol and/or tobacco. The published results from these studies have generally suggested that women who regularly consume alcohol may be at a slightly increased risk of the disease (an increase of 7.1% in the relative risk of breast cancer for each additional 10g per day alcohol), but the findings reported for tobacco are inconsistent (Collaborative Group on Hormonal Factors in Breast Cancer 2002). There is also accumulating evidence that regular intakes of moderate amounts of alcohol affect sex hormone levels, therefore alcohol consumption can have an indirect relationship to breast cancer risk.

Evidence is growing that low folate status may be a factor in the aetiology of several cancers, including breast cancer (Kim 1999). There are two mechanisms by which folate deficiency could increase risk of malignancy: by causing DNA hypomethylation and proto-oncogene activation; and/or by inducing uracil misincorporation during DNA synthesis, leading to faulty DNA repair, DNA strand breakage and chromosome damage (Duthie 1999).

For the most part breast cancer incidence suggests a lifestyle cause (McGrath 2003). Most of the lifestyle choices in question either improve quality of life or has a role in the emotional wellbeing of woman. This is especially true for breast cancer risk factors like oral contraceptive use, hormone replacement therapy, smoking and alcohol consumption. None of these risk factors are going to vanish,

nor even importantly decrease on the grounds of possible attributable increases in breast cancer risk and will therefore remain an enduring problem for women wishing to avoid breast cancer.

2.7 BREAST CANCER SCREENING AND PROGNOSIS

Surveillance programs are essential, especially for women who have an increased risk of developing breast cancer, as the disease is progressive and earlier detection and timely treatment can alter its natural course. Traditional surveillance guidelines for breast cancer would include monthly breast self-examination, starting in early adulthood, and annual or semi-annual clinical breast examination and mammography beginning at age 25-35 years. The limited sensitivity of mammography and an interest in methods of screening that do not involve ionizing radiation have led to evaluation of other screening techniques, including magnetic resonance imaging (MRI), breast ultrasound, breast ductal lavage, and digital mammography. The development of these techniques may lead to further progress, especially in younger women whose breast density may prevent adequate screening by conventional mammography or in those at particularly high risk of developing breast cancer (Stoutjesdijk *et al.* 2001). The availability of genetic testing to identify carriers of *BRCA1* or *BRCA2* mutations raises essential questions about optimal screening and preventive measures for breast cancer.

The distinct pathologic features of *BRCA1*- and *BRCA2*-related tumours coupled with the relatively low frequency of somatic *BRCA* mutations suggest a specific pathogenetic basis, which could lead to differences in prognosis. Most available data are, however, based on small numbers (<50 cases) and are probably confounded by different biases and lack of appropriate controls. El-Tamer *et al.* (2004) found patients with *BRCA1*and *BRCA2* mutations to have a similar outcome as non-mutation carriers, although *BRCA* mutation carriers had a higher incidence of bilateral disease. There was no difference in five- or ten year breast cancer-specific survival between mutation and non-mutation carriers.

2.8 TREATMENT

There are two ways of treating breast cancer, with local therapies and with systemic therapies. Local therapy targets the breast area and the lymph nodes involved though surgery and radiation. With systemic therapy the whole body is treated through chemotherapy, hormone therapy or immune therapy. A treatment plan usually involves a combination of these.

2.8.1 Surgery

Breast conserving surgery (lumpectomy) involves removal of only the tumour and the marginal breast tissue. In a total mastectomy all of the breast tissue is removed, while a modified radical mastectomy refers to removal of the breast as well as the lymph nodes. During a radical mastectomy the underlying chest muscle will also be removed. Bilateral prophylactic mastectomy has shown a reduction in risk for breast cancer in women with *BRCA1* and *BRCA2* mutations by 90% or more (Hartmann *et al.* 2001; Meijers-Heijboer *et al.* 2001; Rebbeck *et al.* 2004).

2.8.2 Radiation therapy

Radiation therapy could be administered by means of high levels of external x-rays directed towards the tumour to damage or destroy the cancer cells. Radiation could also be performed through internal radiation which is administered by implanting a radioactive substance inside the body, close to the tumour. This delivers very intense radiation to a small area of the body and limits the dose to affected tissue (Abramson Cancer Centre of the University of Pennsylvania www.oncolink.org).

2.8.3 Chemotherapy

Patients with high grade, hormone-receptor negative tumours with indications of lymphatic or vascular invasion are treated with chemotoxic agents, directed towards rapidly growing cells (Your guide to breast cancer treatment, developed by www.breastcancer.org). More than one of these agents would be prescribed at a time in different combinations, e.g. CMF (combination of Cytoxan, Methotrexate and 5-Fluorouracil) or AC±T (Adriamycin, Cytoxan and Taxol or Taxotene).

2.8.4 Hormone therapy

Hormone therapy has been the keystone of breast cancer treatment for more than 50 years (Duffy 2005). Hormone therapies include SERMs (selective estrogen receptor modulators), AIs (aromatase inhibitors), ERDs (estrogen receptor down-regulators) and ovarian ablation. A prerequisite for hormone therapy is estrogen- and/or progesterone receptor positive tumours. AIs (e.g. anastrozole, letrozole and exemestrane) reduce the amount of estrogen produced a women's body after menopause.

ERDs (e.g. fulvestrant) block and destroy estrogen receptors. Both AIs and ERDs are prescribed to menopausal women. Ovarian ablation (available to pre-menopausal women), which lowers levels of circulating estrogen, is achieved by surgically removing the ovaries (oophorectomy), radiating the ovaries or with medication, which is only temporary (goserelin acetate is a luteinizing hormone-releasing (LH) hormone agonists). Ovarian ablation has been shown to produce a highly significant improvement in recurrence-free and in overall survival in premenopausal women diagnosed with breast cancer. The benefits appear to be greater in women with estrogen receptor positive primary tumours (Early Breast Cancer Trialists' Collaborative Group 1996).

SERMs are prescribed to both pre- and post-menopausal women. Of these, Tamoxifen is the most established adjuvant treatment (Goldhirch *et al.* 2001). Tamoxifen functions as both an estrogen agonist and antagonist (Shang *et al*. 2000). *BRCA1* tumours are mostly estrogen-/progesterone receptor negative (Karp *et al.* 1997) and *BRCA2* tumours more commonly positive for both receptors (Loman *et al.* 1998). The lack of hormone receptor positivity in *BRCA1* tumours suggests that treatment with tamoxifen, or other hormonal therapy, may be less effective in this patient population (Early Breast Cancer Trialists' Collaborative Group 1998). This drug is also described as a chemopreventive agent, as it decreases the incidence of invasive and non-invasive breast cancer (Fisher *et al.* 1998; Dumitrescu and Cotarla 2005). It has also been suggested that Tamoxifen might reduce breast cancer risk through mechanisms other than receptor-mediated estrogen block (Narod *et al.* 2000).

2.8.5 Immune therapy

The HER-2 protein, which is also known as c-erbB-2 or *neu*, is a member of subclass 1 of the superfamily of receptor tyrosine kinases. Over-expression of the HER-2 gene has been associated with increased mitogenesis, malignant transformation and invasion, increased cell motility and metastasis. Either gene amplification or increased production of HER-2 is generally found to correlate with adverse prognosis (Duffy 2005). Herceptin (chemical name trastuzumab) is an antibody directed against the HER-2 protein.

2.9 THE *BRCA* GENES – *BRCA1* AND *BRCA2*

2.9.1 Cloning of the *BRCA* genes

In 1990, a breast cancer susceptibility gene, *BRCA1*, was localized to chromosome 17q21 (Hall *et al.* 1990). A few years later the gene was positionally cloned using linkage analysis of families with multiple cases of breast and ovarian cancer (Miki *et al.* 1994). Wooster *et al.* (1994) localized the second breast cancer susceptibility gene, *BRCA2*, to a 6cM interval on chromosome 13q12-13. Their preliminary evidence suggested that mutated *BRCA2* confers a high risk of breast cancer (similar to that of *BRCA1* mutations) but, unlike *BRCA1*, does not confer a substantially elevated risk for ovarian cancer. However, they only reported a partial sequence. In the following two years the link between breast cancer and *BRCA2* was confirmed in another study by Wooster *et al.* (1995) and also by Tavtigian *et al.* (1996), who both cloned the gene and determined the complete sequence of the *BRCA2* gene.

2.9.2 Structure of the *BRCA* genes

While *BRCA1* certainly contains some general similarities to *BRCA2*, there are no significant homologies between them, or between *BRCA1* or *BRCA2* and any other characterised gene. Given the differences in primary sequence, the genomic parallels between *BRCA1* and *BRCA2* are particularly striking. Both genes are reasonably large, spanning approximately 70-80kb of genomic DNA, with coding sequences extremely rich in pyrimidines. They both have extremely large central exons, constituting more than 60% of the respective proteins. Comparison of the amino acid sequence of human BRCA1 and BRCA2 with mouse Brca1 and Brca2 indicates that the respective proteins share approximately 60% identity. Figure 2.2 summarizes the most important binding domains for each protein.

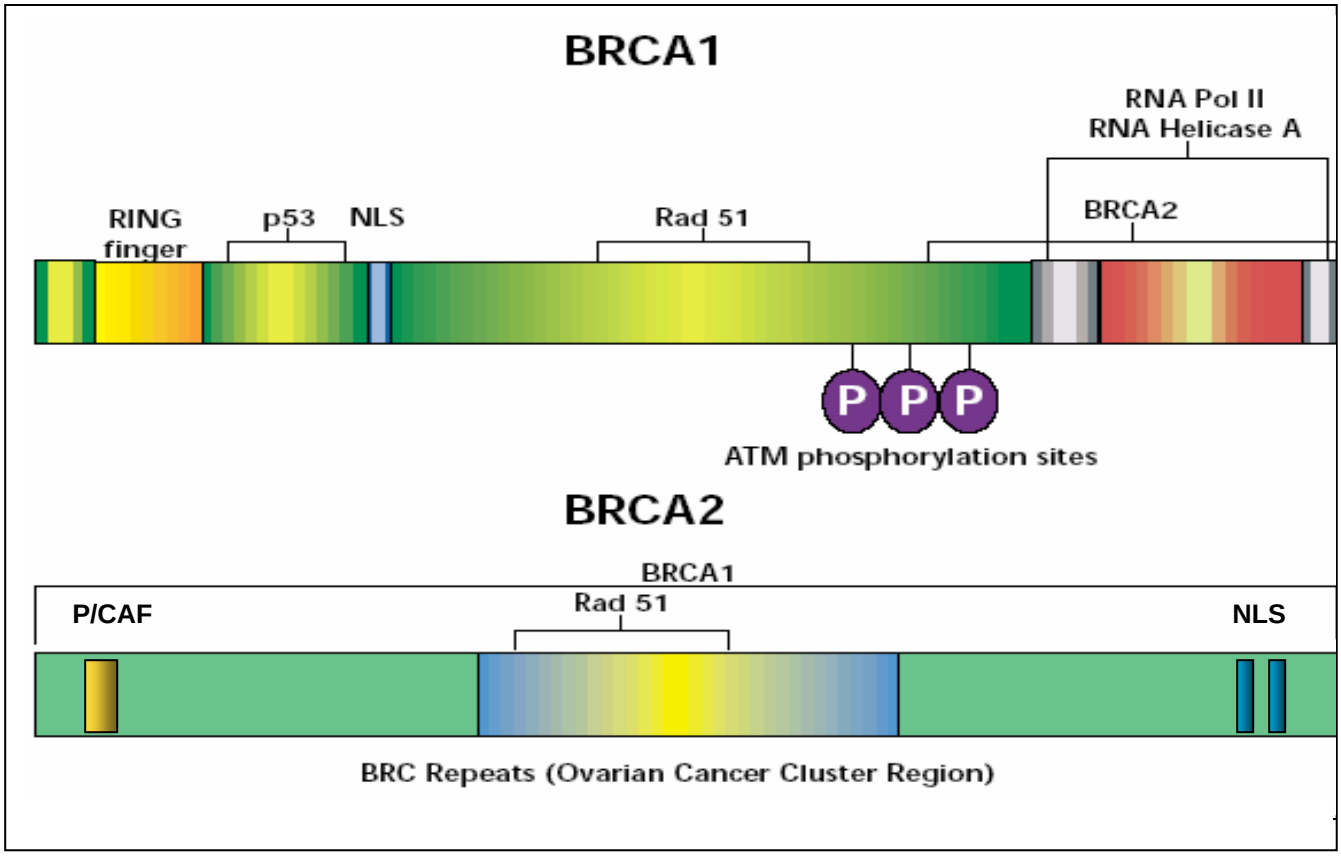

Figure 2.2 BRCA1 protein: The zinc-binding RING finger domain is near the amino terminus, where many protein partners interact. The NLS (nuclear localization signal), p53, RAD51, RNA polymerase II (with the help of RNA helicase A) are indicated as well as the serines phosphorylated by ATM in response to DNA damage (P). **BRCA2 protein*:*** The protein interacts with BRCA1, but a specific domain has not been identified. The RAD51 interaction site is indicated. The central portion containing the BRC repeats has been termed the ovarian cancer cluster region (OCCR). The C-terminus NLSs are indicated as well as the P/CAF binding domain at the N-terminus. Adapted and reprinted with permission from the Nature Publishing Group (Nathanson *et al*. 2001).

2.9.2.1 *BRCA1*

The *BRCA1* gene has 5589 nucleotides of coding sequence. It encodes a 7.8kb transcript which produces a nuclear protein of 1863 amino acids (220 kD). It is distributed over 24 exons, of which 22 are coding. Transcription start from one of two alternatively spliced first exons (exon 1a or 1b) (Xu *et*

al. 1995). Translation starts in exon two and exon four is an intronic *Alu* element. Insertion of exon 4 introduces a premature stop codon, resulting in a truncated protein. Exon 11 is the largest exon and comprises 60% of the coding sequence for the BRCA1 protein. The functional domains suggestive of a role in DNA repair are clustered around exon 11, while motifs involved in transcriptional regulation appear to cluster mainly within the C-terminal BRCT (*BRCA1* carboxyl-terminal) repeats (Hohenstein and Fodde 2003).

The BRCA1 protein is normally located in the nucleus and contains phosphorylated residues (Chen 1996). Two recognizable protein motifs are found. The amino terminus contains a RING finger domain and the C-terminus contains two BRCT repeat domains. It also has two nuclear localizing signals (NLS), a central transcription activation domain which is able to bind DNA, and a RAD51 binding domain (refer to Figure 2.2). RING fingers are known to facilitate protein-protein or protein-DNA interactions, whereas BRCT repeats only facilitate protein-protein interactions. The BRCA1 RING finger specifically interacts with BAP1 (BRCA1-associated protein) and also with BARD1 (BRCA1-associated RING domain), another RING finger protein which was identified based on this interaction (Wu *et al.* 1996). The BRCT repeats are each about 110 amino acids long and comprise amino acids 1653-1736 and 1760-1855. The BRCT domains are usually involved in DNA repair, transcriptional co-activation and cell cycle regulation. Williams *et al.* (2003) found that more than 60% of clinically relevant *BRCA1* mutations delete a portion of or all of the BRCT domains, which highlights the crucial role of the C-terminal. The majority of BRCT missense alterations tested that target the three classes of BRCT folding determinants (BRCT fold, BRCA1 fold, and interface mutations), are destabilizing. The BRCT domain of BRCA1 directly interacts with BACH1 (BRCA1-associated carboxyl-terminal helicase) and with BRIP1 (BACH1-BRCA1-associated C-terminal helicase-1), both members of the DEAH helicase family.

A crucial interaction which BRCA1 has is with RAD51, a key component in homologous recombination and double-strand break repair. The BRCA2 protein also interacts with RAD51, and it is through this mutual association with which it interacts with BRCA1. MYC, p53, RB and ZBRK1 all bind to a region of BRCA1 that includes the NLS. ZBRK1 is zinc-finger protein that suppresses transcription through an interaction with another protein. BRCA1 is required for this repression. SW1/SNF binding occurs between amino acids 260 and 553. The DNA-binding domain encompasses amino acids 452-1 079. It contributes to the DNA-repair-related functions of BRCA1, which are partly

mediated through proteins that make up the BRCA1-associated genome surveillance complex (BASC). This complex includes tumour suppressor and DNA damage repair proteins (MSH2, MSH6, MLH1, ATM, and BLM) and the RAD50-MRE11-NBS1 protein complex. BRCA1 binds the core RNA polymerase II as well as the RNA polymerase II haloenzyme via RNA helicase A (reviewed in Monteiro 2000). This association reveals a function for BRCA1 in transcription.

Jensen *et al.* (1996) discovered a perfect granin consensus in BRCA1 at amino acids 1214-1223. Based on sequence homology, biochemical properties and cellular localization, BRCA1 is proposed to be a member of the granin family (Steeg 1996). Granins are a family of acidic proteins that bind calcium and aggregate in its presence. Granins are located in secretory granules and expressed in neuroendocrine tissues. The breast and ovary are considered to be endocrine glands. The expression of some granin family proteins is regulated by estrogen. Intracellular, granins participate in the regulated secretory pathway. *BRCA1* could therefore encode one or more secreted peptides with autocrine or paracrine functions. Other characteristics of BRCA1 such as estrogen regulation, glycosylation, heat stability and cAMP regulation are all consistent with the granin family, but are not specific for granins.

A *BRCA1* pseudogene, ψ*BRCA*1, had been shown to lie ~30kb upstream of *BRCA1* (Barker *et al.* 1996). The pseudogene is a duplication of a region containing *BRCA1* exons 1a, 1b and exon two. The first exons of the NBR2 (next to *BRCA1* two) gene, located between *BRCA1* and its pseudogene, are homologous to the first exons of the *NBR1* gene (next to *BRCA1* one), which lies head-to-head with ψ*BRCA1* (Xu *et al.* 1997).

2.9.2.2 *BRCA2*

The *BRCA2* gene has an 11385bp transcript that codes for a nuclear protein of 3418 amino acids (384 kD). The *BRCA2* gene is composed of 27 exons, of which 26 are coding. As with *BRCA1*, translation starts in exon two and exon 11 comprises more than 60% of the coding sequence. Like BRCA1, the protein is located in the nucleus and contains phosphorylated residues. If BRCA1 is a granin or granin-like protein, limited sequence similarity suggests that BRCA2 may also serve similar functions. *BRCA2* has recently been identified as identical to the Fanconi anaemia gene (*FANCD1*) (Wagner *et al.* 2004).

The structure of the *BRCA2* gene is not as well characterised as the *BRCA1* gene. One important motif is the BRC repeats, which are conserved across mammals. BRCA2 has eight BRC repeats consisting of 30-80 amino acids, of which four interact with RAD51 (Wong *et al.* 1997). It has two nuclear-localization signals (NLSs) located at the 3' end of the genes, within the final 156 residues (Spain *et al.* 1999). The NLSs are essential for its cellular localization. Mutations predicted to truncate BRCA2 5' to the NLS would render it cytoplasmic and rule out any interaction with the RAD51 complex unless it is transported into the nucleus by alternative means.

Crystallographic studies have revealed that the C-terminal region of BRCA2 contains DNA/DSS1-binding domains (BRCA2DBD) (amino acids 2478-3185), which comprise a helix-turn-helix motif, OB1, OB2, OB3 (oligonucleotide/oligosaccharide-binding) and a Tower region (Yang *et al.* 2002). The function of the DSS1 protein has not been elucidated. Single-stranded DNA (ssDNA) binding has been attributed to two of the three OB folds. The Tower region has been implicated in the interaction with double-stranded DNA (dsDNA). These domains implicate BRCA2 in ssDNA and dsDNA binding. The C-terminal region of BRCA2 (~1000 residues) corresponds to the best-conserved portion of the protein (Warren *et al.* 2002).

The BRCA2 N-terminus interacts with P/CAF (p300/CBP-associated factor) which has histone deacetylase activity. Putative transcriptional activation domains are also located at the N-terminus of BRCA2 (Welcsh *et al.* 2000).

2.9.3 Expression of the *BRCA* genes

2.9.3.1 Tissue specificity of cancer predisposition

The *BRCA* genes are ubiquitously expressed in most adult tissues and cell types. Transcription is up-regulated at the G1/S phase junction of the cell cycle, with expression being maintained at relatively high levels when cells progress through the S and G2/M phases (Wang *et al.* 1997). Despite this ubiquitous expression, germline mutations in *BRCA1* and *BRCA2* predispose individuals to breast and ovarian tumours with only minor effects on the predisposition to cancer at other sites (refer to section 2.9.5), indicating that gene expression does not account for the tissue-restricted phenotype of breast

(and ovarian) cancer. Several hypotheses (discussed in Monteiro 2003) are entertained as to the tissue specificity of cancer predisposition associated with *BRCA* gene mutations.

One possibility for tissue specific cancer predisposition is that in unaffected tissues there might be functional redundancy whereby other proteins perform the same function as the BRCA proteins, but in ovary and mammary gland these other proteins are not expressed at an adequate level. Another suggestion is that BRCA1 and/or BRCA2 disruption may make breast cells more sensitive to the effects of local mutagens, such as estrogen metabolites. Mammary gland tissues do have increased levels of carcinogenic estrogen metabolites that can adduct DNA. Considered from the perspective that heterozygosity for *BRCA* mutations is sufficient to predispose to cancer, an alternative hypothesis is that the frequency with which loss of the second *BRCA* allele occurs may effectively be higher in certain tissues. Breast tissue experience prolonged proliferative dormancy (when BRCA1 and BRCA2 function may be dispensable for viability) alternating with periodic bursts of proliferation (during puberty and pregnancy). It has been shown that expression is upregulated during puberty and pregnancy, when estrogen levels are dramatically increased (Welcsh and King 2001), which suggest that estrogen might stimulate expression of *BRCA1* and/or *BRCA2*. These periods of high proliferation may favour the accumulation of cells in which both *BRCA* alleles have been lost. Unlike the cells of many rapidly proliferating epithelia, such as those of the intestine or of the uterine endometrium, progeny of this proliferative burst are retained within the breast epithelium. Having a larger pool of mutant cells from which tumours could eventually evolve may be of particular significance in the light of the deleterious effects of homozygosity for *BRCA1* and *BRCA2* mutations.

Elledge and Amon (2002) proposed a hypothesis to explain *BRCA1* tissue-specificity by suggesting a delayed apoptotic response in the breast and ovary. It is based on the idea that the loss of BRCA1 function leads to apoptosis or severe proliferation defects in tissues other than breast and ovary, therefore preventing the accumulation of additional mutations required for tumour formation. Temporary suppression of lethality in breast and ovary cells lacking BRCA1 would allow sufficient time to accumulate additional mutations required for oncogenesis. The balance between proliferation and apoptosis is tightly maintained in the mammary gland and cells undergo apoptosis after each estrogen cycle, suggesting that at least some cells are susceptible to apoptosis (Anderson 1999). The apoptotic response may be controlled by tissue-specific factors. Warren *et al.* (2002) propose that certain cell types, particularly those susceptible to tumorigenesis in human *BRCA2* mutation carriers,

might be particularly sensitive to BRCA2 dosage leading to impaired fidelity of DNA repair. The same could be true for BRCA1. This gene-dosage effect is known as haploinsufficiency, whereby one defective allele would be enough to lead to tumorigenesis (Fodde and Smits 2002).

Lastly, since their discovery, BRCA1 and BRCA2 have been implicated in a wide variety of functions (refer to section 2.9.4). Thus, they could perform specific functions in certain tissues with the function performed in breast and ovarian tissues being the only one relevant for tumour suppression. One possibility would be by modulating the activity of estrogen receptor α (ER-α). It has been suggested that BRCA1 can mediate ligand-independent repression of the receptors for estrogen and progesterone. Analysis of *BRCA1* tumours has, however, revealed that these tumours are mostly ER-α negative (Loman *et al.* 1998). It remains unclear whether these tumours originate from ER-α negative cells or whether receptor expression is lost during tumour development.

All the present hypotheses are valid, with no overwhelming evidence for or against any of them. Many of the concepts underlying these various hypotheses are not mutually exclusive and tissue specificity is likely to be explained by a combination of several factors.

2.9.3.2 Tumour pathology

Various research groups (Lakhani and The Breast Cancer Linkage Consortium 1997; Lakhani *et al.* 1998) have characterized the histopathological features of breast cancers in patients harbouring germline mutations in *BRCA1* and *BRCA2*. Their results show that the histological characteristics of breast cancers due to *BRCA1* and, to a lesser extent *BRCA2* mutations, differ from those of sporadic breast cancers, and that they even differ from each other.

Breast cancer in patients with *BRCA1* mutations are of higher grade and have higher mitotic counts, a greater degree of nuclear pleomorphism and less tubule formation than age-matched sporadic breast cancers unselected for family history. *BRCA1* carriers also have an excess of medullary and atypical medullary cancers. However, multifactorial analysis demonstrated that many of the factors mentioned are associated with each other. Tumours are more likely to be estrogen receptor-negative and progesterone receptor-negative (Verhoog *et al.* 1998; Lakhani *et al.* 2002). Information regarding

BRCA2-related tumours is more limited. These tumours are also of higher overall grade as a result of exhibiting less tubule formation, but are not significantly different from controls with respect to mitoses and pleomorphism. Both the reduction in tubule formation and the presence of continuous pushing margins are significantly associated with *BRCA2*. *BRCA2* tumours are at least as likely to be hormone receptor-positive as control tumours of nonhereditary breast cancer (Lakhani *et al.* 2002).

It is of major importance to define the morphological, immunohistochemical, and molecular features of the group of *BRCA1* and *BRCA2* tumours to improve genetic testing and also to gain further insight into the biological characteristics of these tumours.

2.9.4 Functions of the BRCA proteins

2.9.4.1 Introduction

The biological basis of the cancer predisposition in *BRCA1* and *BRCA2* mutation carriers has not been clearly elucidated. Although these genes are classic tumour suppressor genes in that the loss of both alleles has generally been believed to be required for the initiation of malignancy, information on which cellular pathways are affected by alterations in the *BRCA* genes is limited. The observed distinction between *BRCA1 versus BRCA2* deficient tumours emphasizes that cancer evolution proceeds down different routes in each group, consistent with the arguments that the BRCA proteins perform distinct functions in overlapping biological processes.

Cancer susceptibility genes fall into either of two general classes (Kinzler and Vogelstein 1997): (1) Genes whose altered expression relieves normal controls on cell division, proliferation, cell death and lifespan have been termed “gatekeepers”. Their alteration therefore results in the uncontrolled cellular proliferation that characterizes tumour cells. (2) Those genes whose disruption causes genome instability, increasing the frequency of alterations in gatekeeper genes, work instead as “caretakers.” Genes in the caretaker class are involved in DNA metabolic processes and are responsible for maintaining the overall stability of the genome. Collectively findings have established that the BRCA proteins mainly behave as caretakers, but they also display functions relating to gatekeepers. BRCA1 and BRCA2 have similar, but distinct functions in DNA damage sensing, DNA damage repair, checkpoint control and transcription, playing roles to contribute to the stability of the human genome.

BRCA1 is also involved in X-chromosome silencing (Ganasan *et al.* 2002). Both BRCA1 and BRCA2 are essential for cellular development since nullizygous *BRCA1* or *BRCA2* embryos die around the time of gastrulation (Ludwig *et al.* 1997).

Most functions of the BRCA proteins have been discovered through the interactions they have with proteins of known function. These functions are then confirmed by observing the response or behaviour of BRCA deficient cells. Holt *et al.* (1996) provided the first direct evidence for a growth inhibitory role for BRCA1. The authors did not only show that breast or ovarian cancer cell lines transfected with wild-type *BRCA1* are less able to form tumours, but also that BRCA1 can inhibit the growth of established tumours. BRCA1 could not inhibit growth of colon or lung cancer cell lines, nor could it inhibit normal fibroblasts, which provide a clear indication of the specificity of *BRCA1* mutations to breast and ovarian cancer, suggesting these cells have a distinct growth control pathway. Inhibition of *BRCA1* expression does not lead to growth acceleration of only malignant cells, but also of normal breast epithelial cells (Thompson *et al.* 1995).

The complicated network of events in which BRCA1 and BRCA2 are involved in as well as the various interactions these proteins have with other proteins are graphically presented in Figure 2.3. A detailed discussion of these processes and interactions will follow.

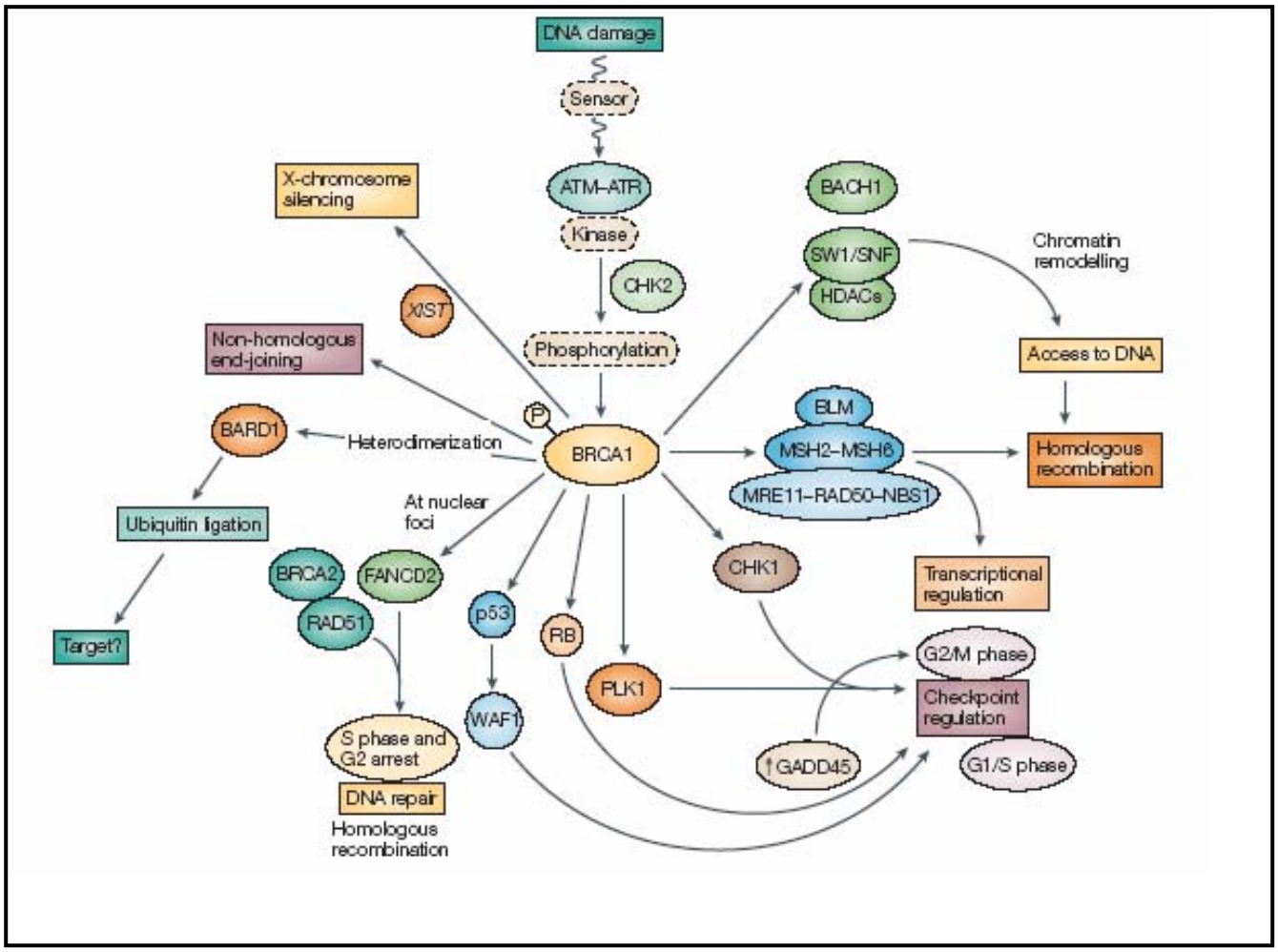

Figure 2.3 The BRCA1 network. Protein-protein interactions with BRCA1 are shown, as well as downstream events in the DNA damage signalling and repair pathway. BRCA1's relationship with BRCA2 and CHEK2 has also been indicated. Reprinted with permission from the Nature Publishing Group (Narod and Foulkes 2004).

2.9.4.2 DNA damage sensing

BRCA1 is rapidly phosphorylated after DNA damage has occurred in dividing cells, suggesting that it may work downstream of the checkpoint mechanisms that sense and signal DNA damage or problems with DNA replication. BRCA1 is phosphorylated by three kinases, including ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and RAD3 related) and CHEK2. Phosphorylation of BRCA1 by each of these kinases is activated by distinct stimuli and is targeted to distinct clusters of

serine residues, suggesting that it will serve a distinct purpose in each instance. The chemical nature of the initiating DNA lesion is an important influence, with differences apparent among ionizing radiation (which primarily induces DNA breakage), UV light (nucleotide lesions), or replication arrest induced by hydroxyurea (strand gaps). ATM and CHEK2 phosphorylate BRCA1 after ionizing radiation (IR) (Cortez *et al.* 1999), whereas ATR is more specifically activated after UV treatment or replication arrest (Tibbetts *et al.* 2000). BRCA1 could therefore be seen as a signal processor that coordinates DNA damage-sensing mechanisms with the appropriate biological response. It is not known if BRCA2 is a target of checkpoint kinases, but its behaviour changes when dividing cells undergo replication arrest or when there is a response to DNA damage (Chen *et al.* 1998).

BRCA1 can exist as part of the BASC complex (Wang *et al.* 2000), indicating a role as a kind of platform for DNA surveillance complex building. All members of this complex have roles in recognition of abnormal DNA structures or damaged DNA, suggesting that BASC may serve as a sensor for DNA damage.

2.9.4.3 Checkpoint control

BRCA1 is hyperphosphorylated during the G1 and S phases of the cell cycle and has been associated with the CDK2-cyclin complex, which controls the G1-S transition. BRCA1, phosphorylated by Aurora-A, plays a role in G2 to M transition of the cell cycle (Ouchi *et al.* 2004). BRCA1 also indirectly controls the G2/M checkpoint, by regulating the expression, phosphorylation and cellular localization of Cdc25C (cell cycle-promoting phosphatase) and Cdc2/cyclin B kinase proteins that are crucial for the G2/M transition (Yarden *et al.* 2002). During the M phase BRCA1 is dephosphorylated. It is not known if BRCA2 is a target of S and G2 checkpoint kinases but, as mentioned before, aspects of its behaviour change during replication arrest and there is reason to believe that the changes can be brought about by phosphorylation. Marmorstein and team (2001) discovered that the BRCA2 complex contains a DNA-binding protein called the BRCA2-associated factor (BRAF35). The protein is mainly expressed during mitosis and the BRAF35-BRCA complex is associated with the early phases of mitotic cell cycle progression, when it is able to bind DNA crossover structures arising during recombination. More work will be needed before it becomes clear whether or not BRCA2 is a regulator of cell cycle events, independent of its role in DNA repair. It is possible that the second event in tumorigenesis might in fact involve the inactivation of a checkpoint gene, rather than loss of the

second *BRCA1* or *BRCA2* allele, which is in accordance with Knudson's two hit hypothesis (Knudson 1971).

Although this needs further investigation, some of the protein-protein interactions of the BRCA proteins suggest their involvement in fixing stalled replication forks after an arrest or pause during normal cell growth. Lomonosov *et al.* (2003) proposed that in BRCA2 deficiency the breakdown of replication forks triggers spontaneous DNA breakage, leading to mutability and cancer predisposition.

2.9.4.4 Chromosomal instability through inappropriate DNA double-strand break (DSB) repair

DNA double strand breaks (DSBs) are repaired through mainly three mechanisms. Homologous recombination (HR) is the method of choice since it is regarded as being error-free. Breaks are repaired in a RAD51 dependent manner through exchange between sister chromatids or between homologous chromosomes. Both non-homologous end joining (NHEJ) and single-strand annealing (SSA) are RAD51 independent and error-prone mechanisms. SSA is a type of HR between short stretches of single stranded DNA at staggered DSBs. Repair is done by pairing *versus* strand exchange.

The major role of BRCA2 in DSB repair is through control of the RAD51 recombinase, while BRCA1 performs a distinct and more general function as a link between the sensing, signalling and effector components of the response to DNA damage (reviewed in Venkitaraman 2002). BRCA2 binds directly with RAD51 (Sharan *et al*. 1997; Wong *et al*. 1997), a protein essential for DSB repair by HR (homologous recombination). It has been shown that BRCA2 bound to DSS1 (BRCA2DBD) stimulates the RAD51-mediated strand pairing and exchange reaction *in vitro* (Yang *et al.* 2002). It is predicted that the release of RAD51 from its sequestered, inactive state must specifically be triggered by DNA damage or replication arrest. The direct role of BRCA2 in the control of RAD51 availability and activity may explain why both SSA and NHEJ are used in a BRCA2-deficient cell to repair site-specific DSBs (Tutt *et al*. 2001). BRCA1 also binds with RAD51 and forms another complex with RAD50, both of which are required for efficient HR, but far less is known about how BRCA1 may work in these reactions. In BRCA1-deficient cells, however, SSA and HR appear to be decreased, and NHEJ predominates as the repair mechanism (Moynahan *et al*. 1999). Therefore, chromosomal instability provoked by a BRCA deficiency is the result of incorrect routing of DSB processing down

inappropriate and therefore error-prone pathways, rather than the failure of repair *per se* (Narod and Foulkes 2004).

Cytokinetic abnormalities have also been linked to hereditary cancer syndromes, characterized by chromosomal instability, and may help to explain why BRCA-deficient tumours are frequently aneuploid (Daniels *et al.* 2004). BRCA1 has been implicated in the control of the assembly of the mitotic spindles and therefore also in the segregation of the chromosomes. BRCA2 may regulate the fidelity of late stages in cytokinesis, but is not an essential component of the machinery for cell separation.

2.9.4.5 Chromatin remodelling

The process of chromatin remodelling facilitates DNA repair by allowing access to the site of damage for the repair machinery. These sites are marked within minutes by the phosphorylation of a histone species, H2AX. BRCA1 is an early migrant to sites of H2AX phosphorylation (Celeste 2002), which follows with several protein-protein interactions. In a study by Bochar *et al.* (2000), researchers isolated the main BRCA1-containing protein complex from cells, and found that BRCA1 is a component of the multiprotein human chromatin remodelling complex SWI/SNF, mediating the ability of BRCA1 to function as a transcriptional coactivator. BRCA1 also interacts with the MRE11/RAD50/Nbs1 protein complex (Zhong *et al*. 1999; Wang *et al*. 2000). MRE11 encodes a nuclease, which opens double stranded DNA to form single-stranded DNA tracts. BRCA1 will in turn interact with other proteins that remodel chromatin, i.e. regulators of histone acetylation and deacetylation (Yarden and Brody 1999; Pao *et al.* 2000) and with DNA helicases such as BACH1 (Cantor *et al*. 2001). BRCA1 regulates cellular localization and recruitment of BACH1 to sites of repair. These findings indicate involvement of chromatin-remodelling complexes in the pathways regulated by BRCA1.

2.9.4.6 Transcription

Apart from participating in protein complexes that have functions intrinsic to the sensing and signalling of different types of DNA lesions, BRCA1 also has upstream and downstream roles in the response. It works as a sequence-specific transcriptional regulator of genes whose expression affects checkpoint

enforcement and other downstream biological responses. BRCA1 can cause p53 mediated transcriptional activation of p21 cyclin dependent kinase after DNA damage. P21 is a potent suppressor of growth at the G1/S checkpoint. Inactivation of BRCA1 in embryos would lead to the accumulation of DNA damage and in response, p21, through p53, is induced. This would reflect the activation of a DNA damage-dependent checkpoint, resulting in cell cycle delay, which could have catastrophic effects on a gastrulating embryo, leading to "death by checkpoint" (Scully & Livingston 2000). P53 and p21 nullizygosity delays the death of *BRCA1*$^{-/-}$ or *BRCA2*$^{-/-}$ embryos. The same type of response can be seen in adult cells. If loss of BRCA1 function were to occur in a pre-malignant cell wherein key checkpoints have already been inactivated, the aberrant DNA structures resulting from BRCA1 inactivation might be tolerated without cell cycle arrest, promoting neoplastic development.

Another association of BRCA1, supporting its function as transcriptional regulator, is with the protein CtIP (function not known) (Wong *et al.* 1998; Yu *et al.* 1998). BRCA1 has to release this protein to allow the transcription of DNA damage response genes, e.g. *GADD45*, which is upregulated if *BRCA1* is overexpressed. The released CtIP will bind to CtBP, a transcriptional repressor. BRCA1 also binds with ZBRK1 to form a nuclear protein complex, which modulates transcription of DNA damage-inducible genes and acts as a repressor for *Myc*-mediated transcription activation (Zheng *et al.* 2000). This enables cell cycle arrest at G1/S. ZBRK1 can repress *GADD45* transcription in a BRCA1-dependent manner (Yun and Lee 2003). Moreover, BRCA1 interacts with the RNA polymerase II core enzyme, as well as with the RNA polymerase II haloenzyme through RNA helicase A (Anderson *et al.* 1998). BRCA1 deficiency would cause a blockage of the RNA polymerase II transcription machinery. Despite all these known interactions which BRCA1 has, there is no firm evidence that any of these interactions reflect general roles of BRCA proteins in the control of gene expression.

Over-expression of the wild-type *BRCA1* gene has been found to inhibit signalling by the ligand-activated ER-α in various human breast cancer cell lines (Fan *et al.* 1999). The amino-terminus of BRCA1 protein binds directly to the activation function (AF-2) domain of ER-α through an estrogen-independent interaction, and the carboxyl-terminus binds to the DNA to repress the transcription of the receptor, and therefore its proliferative effect. In addition, BRCA1 blocks the expression of two endogenous estrogen-regulated gene products, pS2 and cathepsin D (Fan *et al.* 2001).

BRCA2 associates with P/CAF, which is a transcriptional co-activator which has histone acetyltransferase activity (Fuks *et al.* 1998). This interaction is proof for a transcription activation function of BRCA2.

2.9.4.7 Ubiquitination

Ubiquitination is the process in which proteins are tagged for degradation by the proteasome. Like other RING proteins, the BARD1/BRCA1 complex functions as an E3 ubiquitin ligase with undetermined specificity (Ruffner *et al.* 2001; Kentsis *et al.* 2002). BAP1 is an ubiquitin hydrolase that interacts with BRCA1 (Jensen *et al*. 1998) and can, in turn, remove ubiquitin. Ubiquitination could be a way of regulating BRCA1 levels in the cell (Choudhury *et al.* 2004).

2.9.5 Other cancers associated with the *BRCA* genes

In addition to breast and ovarian cancer, individuals with *BRCA1* or *BRCA2* mutations also have an increased risk of other epithelial cancers, for which precise estimates are unavailable. *BRCA*1 mutation carriers suffer increased risks of colon and prostate cancer, suggesting a lifetime risk of 6% for colon cancer in *BRCA1* mutations carriers and 8% for prostate cancer (Ford *et al.* 1994; The Breast Cancer Linkage Consortium 1999). Pancreatic cancer has shown a link with *BRCA2* mutations (Schutte *et al.* 1995) and is often observed in breast cancer families. Cancers of the larynx, oesophagus, colon, stomach, gallbladder and bile duct, haematopoietic system, as well as malignant melanomas have to a lesser extent been observed in *BRCA2* families (Easton *et al.* 1997; The Breast Cancer Linkage Consortium 1999). The more general increase in cancer risk appears to contrast with *BRCA1*, where no excess risk has been observed except for prostate and colon cancer (Ford *et al.* 1994). It should, however, be noted that some of the excess of stomach cancer incidence could be attributed to misclassification of ovarian cancer, and some of the cancers of the gallbladder and bile ducts might have been misclassified pancreatic cancers (The Breast Cancer Linkage Consortium 1999).

2.10 MUTATION ANALYSIS

2.10.1 *BRCA1* and *BRCA2* mutation spectrum

The isolation of the *BRCA1* gene by Miki and colleagues in 1994 and the *BRCA2* gene by Wooster and colleagues in 1995 has prompted a plethora of publications characterizing mutations and polymorphisms in various populations. Several groups worldwide have characterized the spectrum of germline mutations throughout these genes. The Breast Cancer Information Core (BIC) (Friend and The BIC Steering Committee 1995) is an open access online database hosted by the National Human Genome Research Institute (http://research.nhgri.nih.gov/projects/bic). The database was established to catalogue the range and frequency of germline *BRCA1* and later also *BRCA2* mutations. To date several hundred deleterious *BRCA1* and *BRCA2* mutations, polymorphisms and sequence variants with unclassified significance have been published or reported in the BIC database. Mutations are nearly ubiquitously distributed over the coding region with no "hot spots" present (Couch *et al.* 1996a; Couch e*t al.*1996b). The search for mutations therefore requires extensive analysis of the entire coding sequence of both genes.

While a small number of mutations have been found repeatedly in unrelated families, the vast majority have not been reported in more than a few families. Mutations have been identified in various populations which could be traced back to a common ancestor (discussed in section 2.11.6). Of the known *BRCA1* and *BRCA2* mutations, the majority are predicted to result in premature termination of translation, varying in length from 5% to 99% of the full length protein (Couch *et al.* 1996a; Couch *et al.* 1996b). Collectively, mutations consist of small deletions (70%) and insertions (10%) that generate translational frameshift, single base substitutions that directly generate termination codons (10%) and splice site errors (5%) (Rahman and Stratton 1998). Insertions and deletions are mostly only of one or two nucleotides. Pathogenic mutations that cause amino acid substitutions account for a small proportion of *BRCA1* and *BRCA2* mutations (<5%).

Almost all carriers of mutations in *BRCA1* and *BRCA2* are heterozygous and most *BRCA1*- and *BRCA2*-linked tumours have undergone LOH at these loci. Nondisjunction with chromosome loss or with duplication, interstitial and terminal deletion, gene conversion, mitotic recombination between homologous chromosomes and translocations are all mechanisms by which a cell might undergo LOH

(Monteiro 2003). An exception is a woman who was found to be homozygous for *BRCA1* 2800delAA, which results in an in-frame stop codon at nucleotide 2820 (Boyd *et al.* 1995). This allele is expected to encode a 900 amino acid protein. The individual developed breast cancer at age 32, but was developmentally normal. This case is intriguing because it suggests that *BRCA1* may not be essential for normal development and it contrasts with results obtained from complete *Brca*1 nullizygous mice, which die *in utero* (Hakem *et al.* 1996; Ludwig *et al.* 1997). A double *BRCA1* heterozygote, *BRCA2* heterozygote has also been reported. This individual was developmentally normal but developed breast cancer at age 48 and ovarian cancer at age 50 (Ramus *et al.* 1997).

2.10.2 Chromosomal rearrangements: Large deletions and duplications

The genomic sequence of *BRCA1* contains a relatively high percentage of *Alu* repeat elements (41.5% or one per 0.65kb) (Smith *et al.* 1996), compared to the average repeat element content of the human genome (30.4%). These repetitive elements have been suggested to facilitate intragenic recombination leading to exon deletions or duplications in *BRCA1* (Puget *et al.* 1999a). The genomic sequence of *BRCA2* harbours approximately half as many *Alu*-repeats as does *BRCA1*, but still these elements occur on average every 1.5kb. Large genomic deletions in *BRCA1* are infrequent, accounting for only 5-10% of all genomic mutations, and these mutations are probably even less common in *BRCA2* (Puget *et al.* 1999a). Even though large gene rearrangements are rare, any attempt to provide a comprehensive mutation screening strategy for *BRCA1* and *BRCA2* should include a method for the identification of this type of mutation, which would be missed by conventional PCR-based mutation detection methods (Robinson *et al.* 2000). These alternative methods would include Southern blotting or long-range PCR, or as Robinson *et al.* (2000) suggest, a semiquantitative PCR-based fluorescent assay.

Petrij-Bosch *et al.* (1997) identified three large *BRCA1 Alu*-mediated deletions in Dutch cancer patients accounting for 36% of all pathogenic mutations identified in this population (discussed in the section on founder mutations 2.11.6). It has also been demonstrated that *BRCA1* genomic deletions account for more than a third of the pathogenic *BRCA1* mutations in Italian breast cancer families (Montagna *et al.* 2003). Researchers have discovered deletions which removed *BRCA1*'s non-coding exons 1a and 1b (promoter region) as well as exon two which contains the transcription start site (Swensen *et al.* 1997; Brown *et al.* 2002; Puget *et al.* 2002). This deleted area is then replaced with the pseudogene which is a duplication of exons 1a, 1b and exon two. Various other large deletions encompassing one or more

exons have been reported (Puget *et al.* 1997; Puget *et al.* 1999a; Unger *et al.* 2000; Tancredi *et al.* 2004; Ward *et al.* 2005). It has been suggested that large *BRCA1* deletions represent 8-12% of disease causing mutations in American families (Puget *et al.* 1999a; Unger *et al.* 2000).

Another type of *BRCA1* rearrangement has been detected in breast cancer families. Puget *et al.* (1999b) identified a large duplication, involving exon 13 and 6kb of intronic sequence. They demonstrated that a founder effect is likely, but that the mutation is relatively old and will therefore be found in families around the world (also see section on founder mutations 2.11.6). The exon 13 duplication have been shown to be present in 11% of breast cancer patients residing in the UK (Robinson *et al.* 2000).

Only a few large genomic rearrangements in *BRCA2* have been published thus far. One of these involves the insertion of an *Alu*-element into exon 22 which resulted in alternative splicing and exon skipping (Miki *et al.* 1996). Another pertains to a 5kb genomic deletion encompassing exon three, but it is unclear whether the deletion is caused by *Alu*-recombination (Nordling *et al.* 1998). More recently Tournier *et al.* (2004) identified three large *BRCA2* rearrangements in male breast cancer families, i.e. a deletion of exons 12 and 13, a duplication of exons one and two and a complete deletion of *BRCA2*. Five novel rearrangements have been identified in breast cancer patients from Australia and New Zealand (Woodward *et al.* 2005), with *BRCA1* arrangements identified in 2.2% of cases and *BRCA2* in 2%.

2.10.3 Mutations affecting *BRCA1* and *BRCA2* expression

It has been estimated that mutations that affect expression, splicing or stability of the transcript may account for 15-20% of all *BRCA1* and *BRCA2* mutations (Szabo and King 1995). The promoter region of *BRCA1* has been screened in only a few published studies and even less data is available on the promoter region of *BRCA2*. However, to date, results indicate that mutations in regulatory regions of the *BRCA1* and *BRCA2* genes are rarely expected to be the basis of genetic predisposition to breast and ovarian cancer (Brown *et al.* 2002).

2.10.4 Polymorphisms and unclassified variants

The presence of multiple polymorphisms suggests that the development of DNA based screening tests will be technically challenging. False-positive results may occur because of the difficulty in distinguishing between benign polymorphisms and pathological mutations.

Three basic methods are available to determine the clinical significance of these mutations (Shattuck-Eidens *et al.* 1997): (1) family studies to determine whether the mutation segregates with breast cancer in family members; (2) allele frequency analysis to determine whether the allele has a higher frequency in cancer patients than in the general population; and (3) protein function assays to measure the effect of the mutation on the protein.

2.10.5 Variation in risk related to specific mutations

From the viewpoint of genetic counselling, it is important to determine whether different mutations are associated with variable risk. Diez *et al.* (1997) reported an identical twin pair with a family history of breast and ovarian cancer. A *BRCA1* pathogenic mutation, causing a severely truncated protein, was identified. One sister developed breast cancer when she was 32 and ovarian cancer when she was 39 years old. At the time of the study they were 49 years old. Her sister remained healthy. Both had similar reproductive histories and both had a similar lifestyle. This is just one example of the variable penetrance of *BRCA1* mutations against an identical genetic and similar environmental background.

Although some research studies have suggested differences in cancer risk associated with different *BRCA1* mutations, no definitive data is available yet. However, it is worth noting that the ratio of ovarian cancers to breast cancers has been reported to be higher in families with truncating mutations toward the 5’ end of the *BRCA1* coding sequence than in families with mutations close to the 3’ end (Gayther *et al.* 1995). The junction between these two areas is best characterized as a sharp demarcation point (rather than a gradual transition region) located between codons 1435-1443. Thompson *et al.* (2002a) found that a division of *BRCA1* into three regions, of roughly equal size, with different risks associated with mutations in each region, improve the fit. The ovarian-breast cancer ratio was similar for the 3’ and 5’ regions, but significantly higher in the centre. Although the biological mechanism underlying the genotype-phenotype correlation is not known, this observation

adds weight to the hypothesis that BRCA1 proteins truncated midway through the RAD51-binding domain have bound incompletely and behaves differently from truncated proteins that have either bound correctly or not at all. Overall, the balance of evidence remains in favour of the heterogeneity of risk for different *BRCA1* mutations.

Analysis of *BRCA2* mutation data has provided evidence that the risks of breast and ovarian cancer are related to the position of the mutation: truncating mutations in families with the highest risk of ovarian cancer relative to breast cancer are clustered in a region in the middle of exon 11, bounded by nucleotides 3035 and 6629 (Gayther *et al.* 1997). This region was coined the "ovarian cancer cluster region" or the OCCR. Thompson and Easton (2001) found that the risk of breast cancer for mutations inside the OCCR was lower than that for other *BRCA2* mutations (52.3% risk outside *versus* 33% risk within for breast cancer and 11% outside *versus* 20% within for ovarian cancer). The distinct phenotype associated with OCCR mutations relative to other *BRCA2* mutations appears to be predominantly a reduced risk of breast cancer rather than an increased risk of ovarian cancer. It is interesting that most of the families with more than one case of male breast cancer had mutations outside the OCCR. Conversely, male carriers of *BRCA2* mutations that lie outside the OCCR have been proposed to have an increased risk of developing prostate cancer (Thompson and Easton 2001). The observation that at least six of the RAD51-binding motifs, the BRC repeats, lie within the boundaries of the OCCR defined in this study suggests that they may play a role in the variation in cancer risk observed. Risch *et al.* (2001) found that apart from ovarian and prostate cancer, colorectal, stomach and pancreatic cancer occurred only when mutations were in the OCCR.

2.11 *BRCA1* AND *BRCA2* PENETRANCE

2.11.1 Autosomal dominant inheritance of breast cancer predisposition

More than a decade before the cloning of the *BRCA* genes, segregation analysis of families selected for high risk suggested that an autosomal dominant gene influenced risk of premenopausal breast and ovarian cancer, another gene (with different penetrance) influenced postmenopausal breast cancer, and a third (combined with environmental and/or polygenic effects) influenced childhood tumours and early-onset breast cancer (Go *et al.* 1983). The first complex segregation analysis of a population-based series of families indicated that an autosomal dominant allele with high penetrance could fully

explain clustering of breast cancer in families, but that genetic susceptibility was present in only 4% of the families (Newman *et al.* 1988). The majority of studies hereafter, prior and after the cloning of the *BRCA1* and *BRCA2* genes, confirmed these result (refer to Table 2.1). The proportion of breast cancer cases attributable to inherited susceptibility is much higher for early-onset breast cancer (Claus *et al.* 1991; Claus *et al.*1996).

Table 2.1 Summary of the literature addressing inherited susceptibility to breast cancer.

Percentage of Breast Cancer Attributable to Inherited Susceptibility					
Newman *et al.* (1988)	Claus *et al.* (1991)	Colditz *et al.* (1993)	Claus *et al.* (1996)	Struewing *et al.* (1996)	Szabo and King (1997)
4%	2.5%	2.5%	7%	10%	6-10%

2.11.2 Lifetime risk of developing breast cancer

Risk can be presented in more than one way, e.g. odds ratios, lifetime risk, annual risk per 100 000 women, risk at a certain age or risk for a specific time period (Sauven 2004). The lifetime risk of developing breast cancer is usually defined as the risk up to the age of 70 years. Age-specific penetrance refers to the likelihood that breast cancer will develop in a carrier of a predisposing mutation by a certain age. For adult onset diseases, penetrance is usually quoted in conjunction with age and gender components.

It was initially proposed that female *BRCA1* mutation carriers have a lifetime risk of either breast or ovarian cancer of close to 100% (Ford *et al.* 1994). With only one breast cancer susceptibility gene identified at the time, the observed patterns of breast and ovarian cancer risks were presumed to be explained by two different *BRCA*1-susceptibility alleles, one conferring a lifetime breast cancer risk of 91% and the other conferring a lifetime risk of 70%. The second susceptibility "allele" Ford *et al.* (1994) referred to was most likely *BRCA*2.

An individual's risk to develop breast cancer can be stratified for different age groups. Estimates for *BRCA1* and *BRCA2* mutation carriers at age 50 and age 70 years (also referred to as lifetime risk) are

compared in Table 2.2. These results indicate that the lifetime breast cancer risk in *BRCA*2 mutation carriers is comparable to that for *BRCA1*. Antoniou *et al.* (2003) did a meta-analysis that combined data from 22 studies in which patients from the US, UK, Australia and Europe were unselected for a breast cancer family history. The average cumulative risk was 65% by age 70 years in *BRCA1* mutation carriers and 45% in *BRCA2* mutation carriers. The average risk of breast cancer (and also ovarian cancer) is therefore higher in *BRCA1* mutation carriers than in *BRCA2* mutation carriers, as can be deduced from Table 2.2. Relative risks of breast cancer declined significantly with age for *BRCA1* mutation carriers, but not for *BRCA2* mutation carriers.

In men, *BRCA1* does not appear to be associated with a significantly increased risk for breast cancer, although mutations in this gene have been described in affected men (Struewing *et al.* 1995). The most likely interpretation of these findings is that *BRCA1* mutations do confer an increased risk of male breast cancer, but that the risk is lower than the risk conferred by *BRCA2*. The association between *BRCA1* and male breast cancer is thus not clear. The absolute risk of male breast cancer is still small, and many families where the risk of breast cancer is attributable to *BRCA2* will be characterized by female breast cancer only (Wooster *et al.* 1994). Data suggest therefore that *BRCA1* and *BRCA2* mutations do not show the same high penetrance values in male carriers as it does in female carriers. *BRCA2* mutations are responsible for between 8% and 14% of the observed breast cancer risk in male patients (Couch *et al.* 1996a; Friedman *et al.* 1997; Basham *et al.* 2002).

The penetrance of *BRCA* mutations is still a matter of intense research more than 10 years after the cloning of these genes. There is still controversy regarding which estimates of penetrance should be used to counsel women with *BRCA*1 and *BRCA*2 mutations, as different studies continue to generate different figures.

Table 2.2 Cumulative lifetime risk for *BRCA1* and *BRCA2* mutation carriers to develop breast cancer.

Study	**Population**	***BRCA1***		***BRCA2***	
		50 yrs	**70 yrs**	**50 yrs**	**70 yrs**
Female Breast Cancer					
Claus *et al.* (1991)	US		92%		
Easton *et al.* (1993)	UK, US, Europe	59%	82%		
Ford *et al.* (1994)	North America, Western Europe	73%	87%		
Easton *et al.* (1997)	Ireland			60%	80%
Schubert *et al.* (1997)	US with European ancestry			32%	67%
Marroni *et al.* (2004)	Italian	27%	39%	26%	44%
Male Breast Cancer					
Easton *et al.* (1997)	Ireland				6.3%
Thompson and Easton (2001)	Western Europe, US, Canada				2.8%

2.11.3 Contralateral breast cancer

Ascertainment of bilaterality is biased in very high-risk families in that some women will choose bilateral mastectomy following their first diagnosis of breast cancer (Friedman *et al.* 1994). The contralateral rate immediately following the first primary is also likely to be inflated by increased surveillance (Peto and Mack 2000). It is important to note that contralateral breast cancer could in fact be misdiagnosed recurrences or metastasis to the contralateral breast rather than new independent primary lesions.

In the largest published study of contralateral breast cancer incidence (Harvey and Brinton 1985) it was found that the rate of bilateral breast cancer was 1% per year in young patients within five years of their initial breast cancer, but subsequent incidence was independent of age at initial diagnosis and remained almost constant at approximately 0.7% per year for more than 20 years. This constant rate stands in contrast to the rate of breast cancer in the general population, which increases sharply up to age 50 and more slowly from ages 50 to 80. For women who did not have an oophorectomy or any chemotherapy, the 10 year risk of contralateral cancer have been estimated to be 43.4% for *BRCA1* mutation carriers and 34.6% for *BRCA2* mutation carriers (Metcalfe *et al.* 2004).

It is likely, that if a particular allele indeed plays a predisposing role in breast cancer development, it would evidently be overrepresented in bilateral cases (Van't Veer *et al.* 2002). Bilateral tumours share the genetic background of the host, a history of exposure to external hazards and probably some essential features of the metabolic environment (Imyanitiv and Hanson 2002).

2.11.4 Family and twin studies

The risk of breast cancer is approximately doubled among women whose mothers had breast cancer diagnosed before the age of 40 years or who have a sister with breast cancer (relative risk of 2.1), and remains elevated even for those whose mothers were diagnosed with breast cancer at the age of 70 years or older (relative risk of 1.5) (Colditz *et al.*1993). The risk in 1st degree relatives is ~0.3-0.4% per year. Loman *et al.* (2003) found that breast cancer risk in first degree relatives of early-onset breast cancer cases are elevated, irrespective of the *BRCA1* and *BRCA2* mutation status, but the cumulative risk by age 50 years is likely to be higher in first degree relatives of *BRCA1* and *BRCA2* mutation carriers compared with first degree relatives of carriers of unidentified breast cancer susceptibility genes.

The annual risk for monozygotic (MZ) twins is 1.31% per year and 0.5% per year for dizygotic (DZ) twins (Peto and Mack 2000). The value for MZ twins would mean ~0.7% increase for each breast, which is similar to the value for contralateral breast cancer. The annual incidence in risk for MZ twins remains relatively constant throughout life (Peto and Mack 2000). Hamilton and Mack (2003) did a case-control study in MZ and DZ twin pairs. They investigated the role of ovarian hormones in breast cancer risk. Their findings suggest that the risk of breast cancer for genetically susceptible women is

determined not by cumulative exposure to ovarian hormones but rather by unusual sensitivity to pubertal hormones (earlier exposure or exposure to high levels). These results could explain the constant age-specific pattern of risk in twins, which is inconsistent with causation by cumulative exposure to hormones.

Comparisons of the concordance of cancer between MZ and DZ pairs of twins provide information on whether the familial pattern is due to hereditary or environmental influences. One research group has found the genetic determinant to be 27%, the shared environmental effect 6% and the non-shared environmental factors accounting for the remaining 67% (Lichtenstein *et al.* 2000). The high incidence among MZ twins of breast cancer cases as well as the fact that this finding is more pronounced in younger cohorts (Ahlbom *et al*. 1997, Lichtenstein 2000), serves to verify the heritability of this disease. The similarity of the observed high and constant age-specific rate to that in the contralateral breast of breast cancer cases provides additional evidence that bilateral disease is largely attributable to the genome rather than the environment.

The most plausible high-risk genotype consists of multiple co-existing susceptibility alleles acting through heightened susceptibility to hormones, and/or defective tumour suppression (Mack *et al.* 2002). Combinations of synergistic genes could presumably provide a purely genetic explanation of the much higher rate of risk in patients' MZ twins than in their sisters of all other ages, but both genetic predisposition and environmental factors (perhaps *in utero*) may have a major role in conferring susceptibility to breast cancer.

2.11.5 *BRCA1* and *BRCA2* mutation carrier frequencies in breast cancer families

From the maximum-likelihood Mendelian model, Newman's group (Newman *et al.* 1988) determined the frequency of the breast cancer susceptibility allele to be 0.0006 (about one in 1500) in the general population. After the identification of the *BRCA1* and *BRCA2* genes, it was determined that the frequency of cancer-predisposing mutations in the Caucasian population is between one in 500 and one in 1500 (Ford *et al.* 1995; Couch *et al.* 1996a; Couch *et al.* 1996b; Whittemore *et al.* 1997; Peto *et al.* 1999). Szabo and King (1997) compared risk estimates for *BRCA*1 and *BRCA*2 mutations from various studies. In all populations the frequency of *BRCA*1 mutations was 1.5 to two-fold higher than the frequency of *BRCA*2 mutations.

Initially, the *BRCA* genes appeared to be responsible for disease in 45% of families with multiple cases of breast cancer only, and in up to 90% of families with both breast and ovarian cancer (Easton *et al.* 1994). It has been shown that the frequencies are in fact much lower. Important variables, which would influence these estimations, are the number of affected individuals in a family for both breast and ovarian cancer, age at diagnosis and the presence or not of male breast cancer cases. Families in which breast cancer has occurred and in which at least one male has been affected have been reported to have a 60-76% chance of carrying *BRCA2* mutations (Ford *et al.* 1998).

Experimental results on the different contributions of *BRCA1* and *BRCA2* mutations to the breast cancer phenotype vary greatly. This is mainly a consequence of the selection criteria used to include patients in a particular study. In order to compare data, studies were divided into groups according to these criteria (Table 2.3). For this purpose, high-risk breast cancer families are considered to have three or more first or second degree relative with breast and/or ovarian cancer, early-onset breast cancer (<35 years at diagnosis), bilateral disease and male breast cancer. Moderate risk breast cancer families have one or two first or second degree relatives with breast and/or ovarian cancer or individuals with three or more distant relatives (third or fourth degree) with breast and/or ovarian cancer. The population-based studies did not take family history or age of onset of the disease into account.

Only a few published studies have concentrated explicitly on male breast cancer. Although *BRCA1* mutations have been identified in male breast cancer patients, *BRCA2* mutations are mainly responsible for the increased risk. Germline *BRCA2* mutations have been identified in men diagnosed with breast cancer regardless of a family history of the disease (Couch *et al.* 1996a).

Table 2.3 *BRCA1* and *BRCA2* mutation carrier frequencies in breast cancer patients from high- and moderate-risk breast cancer families and in patients unselected for a family history of breast cancer.

Study	Population	Method	Carrier Frequencies	
High-risk			**BRCA1**	**BRCA2**
Easton *et al.* 1993	UK	Linkage analysis	39%	
Castilla *et al.* 1994	US	SSCP	16%	
Schubert *et al.* 1997	European	PTT, SSCP		27%
Håkansson *et al.* 1997	Scandinavian	PTT, SSCP	22.6%	11.3%
Vehmanen *et al.* 1997	Finish	PTT, SSCP/HA		8%
Wagner *et al.* 1999	Central European	dHPLC	9%	8%
Ford *et al.* 1998	UK	Linkage analysis, incorporating mutation data	52%	32%
Claes *et al.* 2004	Belgian	PTT, HA, DGGE, MPLA	14%	9.2%
Moderate-risk			**BRCA1**	**BRCA2**
Inoue *et al.* 1995	Japanese	SSCP	10%	
Krainer *et a.* 1997	US	PTT, ASO		2.7%
Couch *et al.* 1997	UK	CSGE	16%	
Greenman *et al.* 1998	UK	SSCP, FCCM	19%	
Malone *et al.* 1998	US	SSCP	6.7%	
Peto *et al.* 1999	UK	CSGE	4.9%	2.3%
Ozdag *et al.* 2000	Turkish	HA	4%	4%
Ottini *et al.* 2000	Italians	PTT, SSCP	3.7%	4.4%
Armakolas *et al.* 2002	Greek	SSCP		17.9%
Kataki *et al.* 2005	Greek	PTT, SSCP	6.8%	9.2%
Population-based			**BRCA1**	**BRCA2**
Langston *et al.* 1996	US	SSCP, ASO	7.5%	
Newman *et al.* 1998	US	PTT, SSCP, ASO	3.3%	
Risch *et al.* 2001	Canadian, European, UK	PTT, DGGE	7.5%	4%

UK – United Kingdom; US – United States; SSCP – single-strand conformation polymorphism; PTT – protein truncation test; HA – heteroduplex analysis; dHPLC –denaturing high performance liquid chromatography; DGGE – denaturing gradient gel electrophoresis; MPLA – multiplex ligation-dependent probe amplification; ASO – allele specific oligonucleotides; CSGE – conformation sensitive gradient electrophoresis; FCCM – fluorescent chemical cleavage of mismatch.

Eeles (1999) constructed a table with combined data from various studies. According to this analysis, the chance of finding a *BRCA1* or *BRCA2* mutation is less than 10% in all single cases of breast or ovarian cancer, ~10% if it is diagnosed before the age of 35 years and between 10% and 30% if there are two breast cancer cases in the family diagnosed before the age of 50 years. The odds are raised to 50% if three breast cancer cases diagnosed before the age of 50 years are present. They proposed that the chance of detecting a mutation is in fact lower because at least 15% of mutations are regulatory, i.e. not in the coding region of the gene which is the area tested, and the genetic screening methods are approximately 80% sensitive.

2.11.6 Founder mutations

A likely reason for the preponderance of a disease in a specific population is a founder gene mutation effect. This occurs when a population is either established by a small number of people or when a population bottleneck occurs that reduces the population to a small number. When the population expands, the mutation in a founder becomes prevalent (Neuhausen 2000). A population or ethnic group refers to individuals having a common national or cultural tradition, referring to origin of birth or descent (as defined by the Oxford Complete Wordfinder 1996). Identification of a genetic mutation in seemingly non-related individuals from the same population does not necessarily indicate a founder mutation, but merely suggest a common ancestor (i.e. an ancient mutation). Various founder and ancient *BRCA1* and *BRCA2* mutations have been identified.

The Ashkenazi Jews are a genetically homogeneous population in which three mutations (185delAG and 5382insC in *BRCA1* and 6174delT in *BRCA2*) account for almost all *BRCA* mutations, with a population frequency of 2% (Struewing *et al.* 1997). Approximately 30% of breast cancer diagnosed under 40 years of age and 10% of those with breast cancer diagnosed after the age of 40 years are caused by these mutations (Abeliovich *et al.* 1997). In Dutch women three large deletions in *BRCA1* account for 36% of all breast cancer cases (Petrij-Bosch *et al.* 1997). One of the deletions encompasses exon 13 and another exon 22. The third deletion comprises exons 13 to 16 (approximately 14kb). In the Netherlands another founder mutation in *BRCA1*, 2804delAA, has been identified (Peelen *et al.* 1997). The *BRCA2* mutation 999delTCAAA accounts for up to 40% of male breast cancer patients in the Icelandic population (Thorlacius *et al.* 1997). Puget *et al.* (1999b) reported a 6kb duplication of exon 13 which creates a frameshift in the coding sequence. The *BRCA1* Exon 13 Duplication

Screening Group (2000) speculates that this duplication is most likely a founder mutation distributed mainly in English-speaking countries or in countries with historical links to Britain. The *BRCA1* 943ins10 mutation, a 10bp tandem duplication in exon 11, was originally identified in the African American population, but could be traced back to the Ivory Coast of West Africa and is possibly more than 200 years old (Mefford *et al.* 1999). Other populations for which founder mutations have been described include the Finns, Germans, Norwegians, Russians, Swedish and French-Canadians (reviewed in Neuhausen 2000).

Reeves *et al.* (2004) screened *BRCA1* in South African breast and/or ovarian cancer families (at least three affected family members with breast cancer ≤ 55 years and ovarian cancer ≤ 60 years of age) of predominantly European descent. Only 20% of their families (15% if Ashkenazi Jewish families are excluded) had pathogenic mutations within the coding region of *BRCA1*, which agrees with figures for moderate- to high-risk families from other populations (refer to table 2.3). These mutations included six different frameshift (185delAG; 448insA; 1127insA; 1493delC; 4957insC; 5382insC), and two distinct nonsense mutations (E881X; S451X). Four of these mutations have not been described previously (S451X; 1493delC; E881X; 4957insC). The E881X mutation was identified in 6% of the study population. Haplotype analysis confirmed that these patients shared a common ancestor. These results show that the majority of Afrikaner mutations either arose independently in this country or are rare in the various countries of origin.

2.11.7 African and African American populations

The epidemiology of breast cancer among women of sub-Saharan Africa appears to be similar to that of African-American women as both groups have relatively low incidence rates, higher mortality rates, increased prevalence of early-onset disease and advanced stage tumours. It has been postulated that the lower postmenopausal breast cancer incidence rates observed for African populations are a consequence of demographics, especially population age and overall life expectancy (Fregene and Newman 2005). Breast cancer case ascertainment may also be lower among older African women. The reproductive patterns within African populations, which include multiparity, initiation of childbearing at younger ages, and prolonged lactation, could contribute towards a lower incidence rate. The available information on the epidemiology of breast cancer in African American women and the contributing genetic factors are summarized in a review from Newman (2005). It is apparent from this

review, that the influences of parity, socioeconomic status, screening rates, and hereditary factors on the ethnicity-related breast cancer risk deserve further study.

Few BRCA1 and BRCA2 studies have been done in any of the African populations or in the African-American population. Almost all studies performed to date have defined breast cancer risks and related genetic factors in Caucasian women of European descent. Earlier studies suggested that inherited *BRCA1* and *BRCA2* mutations might be less common in breast cancer patients of African American ancestry than in other populations (Newman *et al.* 1998). Gao *et al.* (2000) selected African American families according to a minimum eligibility criterion of two cases of breast and/or ovarian cancer among first-degree relatives. They reported *BRCA1* mutations in 21.4% and *BRCA2* mutations in 12.5% of families, which is consistent with mutation frequencies in other populations (refer to table 2.3). It has been proposed that African Americans have a unique mutation spectrum (Olapade *et al.* 2003).

2.11.8 Models for predicting risk

Various probability models have been developed to estimate the likelihood that an individual or family has a mutation in the *BRCA1* or *BRCA2* genes. Four priori probability models (Couch *et al.* 1997; Shattuck-Eidens *et al.* 1997; Frank *et al.* 1998) and BRCAPRO, a computer program that implements a statistical model (Parmigiani *et al.* 1998), are most often referred to. Each has unique attributes determined by the methods, sample size, and population used to create the model. Two other models for predicting breast cancer risk, the Gail (Gail *et al.* 1989) and the Claus model (Claus *et al.* 1994), are also widely used in research studies and clinical counselling. The Gail model is a well validated model but, although it includes epidemiological factors, it does not adequately weigh familial risk factors. The tables published by Claus are also well validated and in a simple pedigree, give a good estimate of risk. However, these tables do not take unaffected relatives into account and in a large family will therefore overestimate the risk in these circumstances, neither do they include paternal relatives or cases of ovarian cancer, both of which may increase risk. Recently, Evans *et al.* (2004) published a new scoring system to calculate the risk of identifying a *BRCA1* or *BRCA2* mutation. Their system assigns scores depending upon the type of cancer and age at diagnosis, such that a total score of 10 is equivalent to a 10% chance of identifying a mutation.

All models have limitations, and the risk estimates derived from the models may differ for an individual patient. They do, however, represent the best methods currently available for individual risk assessment.

2.11.9 Predictive testing

Genetic screening or genetic susceptibility testing are terms widely used and understood in medical practice for the assessment of genetic risk. It is essential for clinicians to be able to select high-risk patients, since so few cases are hereditary. Although computer models and written guidelines exist for predicting the chance of finding a mutation (see previous section on models for predicting risk), none of these include all the information that an experienced clinician will use to make a judgment about genetic testing (Eccles 2003). Risk perception is a complex issue and is not directly related to the absolute level of risk. Women with a family history of breast cancer often over-estimate their personal risk for cancer and may view themselves as candidates for genetic testing even when the likelihood of an informative test result is low (Burke *et al.* 2000). A threshold for mutation analysis has been put at a 10% (US) and at a 20% (UK) likelihood of identifying a mutation (Sauven 2004).

Although clinical evaluation of the *BRCA1* and *BRCA2* genes has been available since 1996, there has been no clear consensus on the specific personal and family history features that should prompt consideration of hereditary cancer risk assessment (Frank *et al.* 2002). Candidates for genetic testing according to Sauven (2004) are:

- single cases of breast cancer at <40 years of age if Ashkenazi Jewish
- two breast cancer cases <40 or three <50 years of age
- four cases of breast cancer at <60 years of age
- > four cases of breast cancer at any age
- ovarian and breast cancer in a family (breast cancer <50 years of age if only one ovarian and one breast cancer case)
- female breast cancer at <60 years of age and male breast cancer at any age

For predictive or diagnostic testing, it is essential to be able to correctly interpret an observed sequence variant to establish the causal role of the variation in the pathogenesis of the disease. Since both *BRCA* genes have a great number of heterogeneous sequence variations, this is a major challenge. Extensive biological assays and/or family studies have to be done to elucidate whether observed variants have a consequence for the protein function. This is particularly true for sequence changes that have not been reported previously. If experimental evidence is not available, the pathological significance of a variation has to rely on plausible considerations. All other variants must be considered as "unclassified" until functional evidence is available.

Predictive testing should only be offered to individuals when accompanied by pre- and post-testing counselling with a qualified genetic counsellor. Because of the heterogeneity of *BRCA1* and *BRCA2* mutations, laboratory testing for all possible mutations is challenging. False-positive results may occur as a result of missense mutations that are not associated with an increased risk for breast cancer. False-negative results may also occur because not all mutations are known. Emphasis should be laid on the fact that a positive result does not indicate if or when breast cancer will develop, but only documents an increased risk of developing the disease (Mosconi and Leccese 2004). Genetic counselling provides an opportunity to convey the limitations of genetic testing for women at risk, including the high likelihood of a non-informative test result.

Several approaches, such as intense or more frequent surveillance programs, lifestyle changes, chemoprevention and prophylactic surgery have been demonstrated to provide a clinical benefit for women who test positive for a *BRCA1* or *BRCA2* mutation or for their relatives (Eeles 1999). Decisions regarding these options must be individualised and psychological support must be offered during the period of decision and follow-up. As regards the reasons for not wanting to be tested, Bruno *et al.* (2004) found in their study that most women could only provide an emotional reply, declaring that there were no specific reasons justifying their reluctance. Reliability of modern medicine and concerns regarding legal and social implications seem to be of secondary importance in determining the reluctance of women to be tested (Bruno *et al.* 2004). Education should focus on scientific fact, but it should encompass psychological, social and ethical aspects.

The evidence as to whether genetic testing has an adverse effect on psychological functioning is varied, with some studies reporting no significant effects on general distress and other studies revealing

psychological difficulties specific to genetic test results (reviewed in Halbert 2004). Negative test results are generally associated with a decrease in depressive symptoms, decreases in anxiety and decreases in cancer-specific distress for individuals who consider themselves to be at an increased risk for developing breast cancer with regards to a family history of the disease.

2.11.9.1 Ethical issues

Genetic testing for breast cancer susceptibility raises ethical and social concerns related to the adequacy of informed consent, the availability and quality of pre- and post-test counselling, and the avoidance of genetic discrimination. It is essential to offer pre-test counselling, both to evaluate the individual's capacity for autonomous decision-making and to provide a realistic view of the risks and benefits, the efficacy and alternatives, the seriousness and potential treatment of disorders, as well as the social and ethical implications involved. It is important to explain that the results of DNA testing have implications not only for the patients, but also for their biological kin. The disclosure of genetic test results can have psychological and economic ramifications not only for the person screened, but also for her (or his) relatives (Durfy *et al.* 1998). Some individuals screened may experience anxiety or other adverse psychological effects or disrupted personal relationships (Coughlin *et al.* 1999), again emphasizing the importance of genetic counselling. Genetic knowledge may either increase the sense of control on a woman's life or it may paralyze the decision-making process by shedding a dim light on the woman's future (Surbone 2004).

Other ethical and social issues concern the cost of and access to genetic tests for *BRCA1* and *BRCA2* mutations, especially among women who are socioeconomically disadvantaged or uninsured. While newly identified, high-risk individuals may be more motivated to undergo genetic testing, payment for genetic counselling and testing may be an important factor in testing decisions.

Finally, an important issue is whether minors should be tested for mutations in *BRCA1* and *BRCA2*, as it is an adult onset disease, which is treatable. For the same reasons and for important consequences with respect to abortion and eugenics, prenatal DNA testing for *BRCA1* or *BRCA2* is also contraindicated (Di Pietro *et al.* 2004).

2.12 OTHER CANDIDATE BREAST CANCER SUSCEPTIBILITY GENES

It has been shown that a number of families with large numbers of early onset breast cancer cases are not linked to *BRCA1* or *BRCA2* mutations. Even if the likelihood of missed mutations and other biases are taken into account, most of the familial risk of breast cancer appears to be due to genes other than *BRCA1* and *BRCA2*. After analysis of three-generation population-based families with breast cancer, Cui *et al*. (2001) concluded that there appears to be residual dominantly inherited risk for breast cancer in addition to those risks derived form mutations in *BRCA1* and *BRCA2*, even when additional recessive or polygenic effects are allowed for. This suggests that there is still substantial dominantly inherited risk not explained by *BRCA1* and *BRCA2*. Support for a major autosomal recessive component to breast cancer susceptibility also exists (Kaufman *et al.* 2003).

2.12.1 The putative *BRCA3* locus

Several candidate regions for a third high penetrance breast cancer gene have been proposed. Suggested regions include chromosome 13q21 (Kainu *et al.* 2000) and chromosome 8p12-22 (Seitz *et al.* 1997a; Seitz *et al.*1997b), but both have been strongly refuted by analysis of data from independent families (Rahman *et al.* 2000; Thompson *et al.* 2002b). More recently the *NuMA* region on chromosome 11q13 has been implicated (Kammerer *et al.* 2005). NuMA is a cell cycle-regulator protein essential for normal mitosis. Another candidate locus which familial breast cancer has been linked to is chromosome 19q13.2. The *ICAM* adhesion molecules have been mapped to this area. Further investigation is needed to confirm these associations.

2.12.2 *BRCA1* and *BRCA2* modifier genes

Modifier loci of *BRCA1* are under investigation, including a possible modifier locus on chromosome 5q (Nathanson *et al.* 2002) and the HRAS1 variable number of tandem repeat (VNTR) locus (Phelan *et al.* 1996; Tamimi *et al.* 2003). Recently a novel gene product, EMSY, has been described (Hughes-Davies *et al.* 2003) which suppresses the transcriptional activity of *BRCA2*. Overexpression of EMSY may mimic the effects of *BRCA2* inactivation.

2.12.3 Low penetrance breast cancer genes

Apart from *BRCA1* and *BRCA2*, all other susceptibility alleles, identified to date, are predicted to be rare in the population, with mutations in these genes accounting for a very small fraction of heritable susceptibility for breast cancer (Nathanson *et al.* 2001). Candidate genes from many cellular pathways have been investigated, including hormone metabolism, carcinogen detoxification, DNA-damage response and immune surveillance. It is unlikely that the final list of breast-cancer susceptibility alleles will be neatly divided into high- and low-penetrance. If mutations in several genes acting in concert account for small subsets of the *BRCA1*- and *BRCA2*-negative families, identifying such genes by linkage analyses and positional cloning will be difficult. Associations are constantly confirmed and refuted. Some of the candidate genes, which have been the most extensively studied and have shown positive association, will be mentioned in the following paragraphs.

Enzymes involved in the metabolism of environmental carcinogens, like the cytochrome P450 proteins, the N-acetyltransferases (NAT1 and NAT2) and the glutathione-*S*-transferases (GSTM1 and GSTT1) have been investigated for polymorphism that may lead to individual differences in susceptibility to breast cancer. The relationship between specific gene polymorphisms and environmental factors has therefore also been explored. Examples of these include possible interactions between NAT1, NAT2, GSTM1 and GSTT1 and diet, alcohol consumption and smoking. These genes are polymorphically expressed in a variety of tissues. Another association which has been reported is between methylenetetrahydrofolate reductase (MTHFR) and folate intake, other dietary factors and alcohol (Sharp *et al.* 2002; Le Marchand *et al.* 2004). The MTHFR protein is involved in folate metabolism, DNA biosynthesis, methylation and genomic integrity in actively dividing cells. Distinct combinations of the X-ray repair cross-complementation group (*XRCC1*, *XRCC2* and *XRCC3*) polymorphisms appear to be associated with either an increased breast cancer risk or the possibility of developing an adverse radiotherapy response seen in some breast cancer patients (Moullan *et al.* 2003).

The role of endogenous and exogenous estrogens in breast tumorigenesis has been discussed in section 2.6. Some of the cytochrome genes, which have been implicated in breast cancer risk, include CYP1A1, CYP1B1, CYP17 and CYP19, all of which are involved in the synthesis and metabolism of estrogen (reviewed in Kristensen and Børresen-Dale.2000). Catechol-*O*-methyltransferase (COMT) is another protein involved in this pathway, regulating the oxidizing of estrogens to catechol estrogen and

estrogen quinines, which have been implicated in breast cancer. Sequence variations in the progesterone receptor (PR), estrogen receptor (ER) and androgen receptor (AR) genes have also been associated with an increased risk for developing breast cancer.

Some of the more recent breast cancer associations which have been reported in the literature include Aurora-A/STK15/BRAK, which is implicated in the regulation of centrosome duplication thereby regulating mitosis (Lo *et al.* 2005), and the vitamin D receptor (*VDR*) (Lowe *et al.* 2005). The *CHEK2* gene, which is a cell cycle checkpoint kinase, has been shown (Meijers-Heijboer and The CHEK2-Breast Cancer Consortium 2002) to be a plausible candidate low-penetrance breast cancer gene and will be discussed in section 2.12.4.

2.12.4 *CHEK2*

2.12.4.1 Gene and protein structure

Cell-cycle-checkpoint kinase two (*CHEK2* [MIM #604373] also known as *CHK2*), mapped to 22q12.1, is a 1731bp cDNA homologue of the yeast G2-checkpoint kinases CDS1 (*Schizosaccharomyces pombe*) and RAD53 (*Saccharomyces cerevisiae*) (Matsuoka *et al.* 1998). The gene, which has 15 exons, encodes a 60kD translation product of 543 amino acids. This nuclear protein is a member of the Cds1 subfamily of serine/threonine protein kinases. Two transcript variants encoding different isoforms have been identified for this gene, as well as variants utilizing alternative polyadenylation signals. Blasina *et al.* (1999) and Chaturvedi *et al.* (1999) independently identified *CHEK2*. The gene has a single kinase domain (residues 226-486) and a fork head-associated domain (residues 115-175) (Matsuoka *et al.* 1998), which is involved in protein-phosphoprotein interactions. These interactions are essential for transmitting DNA damage signals to CHEK2. Any alteration of the association of CHEK2 with upstream signalling proteins could lead to the failure of CHEK2 activation following DNA damage. In addition, the FHA (fork head associated) domain mediates transmitting signals from CHEK2 to downstream effectors such as p53, BRCA1 and Cdc25C.

2.12.4.2 Pseudogenes

A number of regions of the genome exhibit a high homology to the 3' terminal exons of *CHEK2*, possibly pseudogenes, making mutational analysis problematic. This phenomenon was first reported by Sodha *et al.* (2000; 2002a), who did a database search which revealed homologous loci on chromosomes seven, 10, 15, 16, 22 and X. Together these loci encompass exons 10 through 14 of the gene and share 95-98% homology. Further mapping studies (Bell *et al.* 2000) showed additional 3' gene fragments on chromosomes two, 13 and Y.

2.12.4.3 Functions of the CHEK2 protein

Cell cycle checkpoints are biochemical pathways that ensure the orderly and timely progression and completion of critical events such as DNA replication and chromosome segregation. Activation of checkpoints in the G1 and G2 phases in response to DNA damage, in the S-phase upon inhibition of DNA replication, or in mitosis after disruption of the spindle, leads to cell cycle arrest. Such delays provide time for repair processes or, in case of severe damage, for the activation of programmed cell death. Defects in the checkpoint regulatory network result in increased sensitivity to damaging agents and to the genomic instability that is often observed in cancer. CHEK2 is involved both in DNA replication and in DNA damage checkpoints.

CHEK2 is required for blocking the cell cycle in response to DNA damage and other stresses. Phosphorylation and therefore activation of CHEK2 occurs when cells are treated with hydroxyurea, in response to ultraviolet radiation and also through phosphorylation by the ATM protein in response to ionizing-radiation. In return it phosphorylates and inactivates Cdc25C which ultimately results in G2 arrest and prevention of the initiation of mitosis. It also phosphorylates p53, BRCA1 and other proteins (see Figure 2.4). CHEK1 has a similar function to CHEK2. It phosphorylates Cdc25C at the same site as CHEK2.

By phosphorylating p53, the protein is stabilized and its accumulation leads to cell cycle arrest in the G1 phase and ultimately ending in apoptosis. Cells lacking CHEK2 fail to accumulate p53 when necessary. Disruption of p53 is a frequent event in cancer. Tominaga *et al.* (1999) propose the existence of a counter-regulatory mechanism; when cells lose functional p53 and thus G1 checkpoint

control, CHEK2 plays a pivotal role in the DNA damage checkpoint during the G2 phase of the cell cycle. CHEK2 phosphorylates p53 at multiple DNA-damage inducible sites and reciprocally is down-regulated by p53 (Chehab *et al.* 2000). *CHEK2* inactivation is therefore partially equivalent to p53 inactivation, in that CHEK2 deficiency facilitates the development, survival and proliferation of BRCA1-deficient T cells at the expense of genomic integrity (McPherson *et al.* 2004). Phosphorylation of BRCA1 is required for the release of BRCA1 from CHEK2, which is important for the ability of BRCA1 to restore survival after DNA damage (Lee *et al.* 2000). CHEK2 is also capable of phosphorylating Cdc25A, leading to activation of an S-phase checkpoint. Defects in the ionising radiation-induced S-phase checkpoint cause "radio resistant DNA synthesis", a phenomenon that has been identified in cancer-prone patients suffering from ataxia-telangiectasia. *CHEK2* is therefore a tumour-suppression gene.

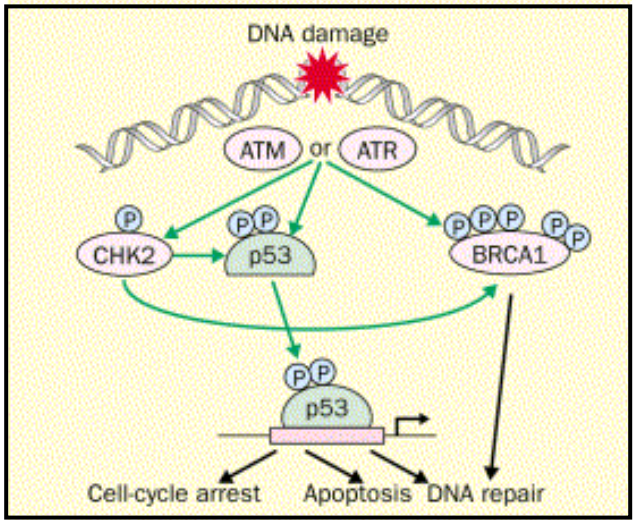

Figure 2.4 Outline of the involvement of CHEK2 in response to DNA damage. Reprinted with permission form Elsevier, The Lancet (Bradbury 2002).

2.12.4.4 *CHEK2* expression

Northern blot analysis done by Matsuoka *et al.* (1998) revealed wide expression of low amounts of *CHEK2* with larger amounts in human testis, spleen, colon and peripheral blood leukocytes. Lukas *et al.* (2001) also addressed the issues of expression and regulation of *CHEK2* in relation to various cell cycle phases and in proliferating *versus* differentiated cells and tissues in their study. Their findings indicated that CHEK2 is a long-lived, predominantly nuclear protein that is constitutively expressed at

low levels but can become activated to increased levels of expression upon DNA damage in all phases of the cell cycle as well as in non-proliferation and terminally differentiated cells. Furthermore, their results indicate that the highest degree of homogenous expression of *CHEK2* is in the cancer-prone, continuously renewing epithelial cell populations exposed to environmental carcinogens, such as the epidermis, the epithelia of the alimentary canal and also in tissues of the breast and endocrine glands, regardless of the proliferation status. CHEK2 is also localized in postmitotic neurons. However, there is a lack of *CHEK2* expression in a few terminally differentiated, static cell populations such as adult skeletal muscle and cartilage. These findings correspond to the predominance of sarcomas, breast cancer, brain tumours, leukaemia and tumours of the adrenal cortex in Li-Fraumeni Syndrome in which *CHEK2* mutations are also implicated.

2.12.4.5 *CHEK2* as a candidate gene for breast cancer and the 1100delC variant

CHEK2 was first linked to cancer susceptibility in 1999 (Bell *et al.* 1999), when patients with Li-Fraumeni syndrome were found to have germline *CHEK2* mutations. The syndrome is characterized by familial clustering of specific cancers, including breast cancer. Three *CHEK2* mutations were found, one of which was the 1100delC variant in exon 10, a frame shift mutation leading to a premature stop at codon 381. Exons 10 to 12 encode part of the kinase domain of the expressed protein, which makes the *CHEK2**1100delC a true disease-causing mutation with the loss of kinase activity (Wu *et al.* 2001).

Meijers-Heijboer and The CHEK2-Breast Cancer Consortium (2002) analysed the *CHEK2* gene and found the *CHEK2**1100delC variant at a frequency of 1.1% in healthy individuals and 5.1% in individuals with breast cancer derived from families that did not carry mutations in *BRCA*1 or *BRCA*2, including 13.5% of individuals from families with male breast cancer. They estimated that the *CHEK2**1100delC variant results in an approximately two-fold increase of breast cancer risk in women and a 10 fold increase risk in men. Walsh *et al.* (2002) evaluated the relative risk the *CHEK2**1100delC variant confers in a population based breast cancer study. The variant accounted for 2% of high-risk *BRCA*1 and *BRCA*2 mutation negative families from North America. A lower frequency (0.45%) was observed among women of Jewish ancestry. This study also included African American breast cancer cases, but the 1100delC allele was not found in any of these individuals. Another research group (Vahteristo *et al.* 2002), reported a frequency of 1.4% among controls, 1.5%

among patients of Finnish ancestry with no family history of breast cancer and a frequency of up to 6.2% among patients with one affected first degree relative.

The *CHEK2**1100delC variant have only been detected in patients who tested negative for *BRCA1* and *BRCA2* mutations (Meijers-Heijboer and The CHEK2-Breast Cancer Consortium 2002; Vahteristo *et al.* 2002; Walsh *et al.* 2002). Therefore, the variant does not confer an increased cancer risk in carriers of *BRCA*1 or *BRCA*2 mutations. It has been suggested that the biologic mechanisms underlying the increased risk of breast cancer in *CHEK2* mutation carriers are already overturned in carriers of *BRCA*1 or *BRCA*2 mutations, which is consistent with participation of the encoded proteins in the same pathway (Meijers-Heijboer and The CHEK2-Breast Cancer Consortium 2002).

2.12.4.6 Other *CHEK2* variants and breast cancer

Various groups screened the entire coding sequence of *CHEK2* in breast cancer patients, still identifying only the *CHEK2**1100delC mutation. The Ingvarsson group (Ingvarsson *et al.* 2002) used microsatellite markers to analyse LOH at chromosome region 22q, where the *CHEK2* gene is located, and screened breast and other tumours for mutations. They found that *CHEK2* inactivation did not play a major role in cancer growth. One of the variants identified, Thr59Lys, could be a low-penetrance allele with respect to breast cancer. In another study three variants, Arg180His, Arg117Gly and Arg137Gln, together with 1100delC, were found to account for 7% of familial breast cancer cases (Sodha *et al.* 2002b). Kuschel *et al.* (2003) determined that two common polymorphisms in the gene, IVS1+38insA and Ala1013Gly, are not associated with breast cancer, but evidence has been found that the Ile157Thr variant may be associated with breast cancer risk (Kilpivaara *et al.* 2004). Recently, in a population based case-control study among North American women, three *CHEK2* variants (1100delC, Arg145Trp and Ile157Thr) have been analysed (Freidrichsen *et al.* 2004). No statistically significant association was observed between any of the *CHEK2* variants and breast cancer risk. Recently an ancient *CHEK2* allele has been identified in the Ashkenazi Jewish population (Shaag *et al.* 2005). This variant, Ser428Phe, which falls within the kinase domain of the protein, was detected at a frequency of 2.88% among breast cancer patients with a family history of breast cancer and at a frequency of 1.37% among controls. This would constitute a ~2 fold increased risk, similar to the risk conferred by the 1100delC variant (Meijers-Heijboer and The CHEK2-Breast Cancer Consortium 2002; Vahteristo *et al.* 2002).

Published data suggest that the *CHEK2* coding sequence is highly conserved and that variants are rare, cryptic or specific to certain populations. The low prevalence and penetrance of *CHEK2* mutations, together with no or an uncertain elevation in risk for other *CHEK2* mutations, suggest a limited relevance for *CHEK2* mutations in familial breast cancer (Allinen *et al.* 2001; Schutte *et al.* 2003; Dufault *et al.* 2004).

2.12.4.7 *CHEK2* and other cancers

Unlike most *BRCA1* and *BRCA2* mutations, the *CHEK2**1100delC variant does not seem to be associated with ovarian cancer (Vahteristo *et al.* 2002). Other *CHEK2* mutations have been identified in lung cancer (Matsuoka *et al.* 2001) and in patients with osteosarcoma and ovarian cancer (Miller *et al.* 2002). Mutations are, however, not common in these cancers. Meijers-Heijboer *et al.* (2003) linked the 1100delC mutation to 18% of families with hereditary breast and colorectal cancer (HBCC). This mutation was, however, not the major predisposing factor but appeared to act in synergy with other susceptibility genes. Dong *et al.* (2003) identified a range of *CHEK2* mutations in prostate cancer cases. Sixteen of these mutations were not present in their control group, suggesting a possible pathological effect of *CHEK2* mutations in prostate cancer development.

2.13 MUTATION DETECTION TECHNIQUES

Numerous techniques have been developed for the detection of single base substitutions and small deletions or insertions. These include direct DNA sequencing (DS), single-strand conformation polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (dHPLC) and heteroduplex analysis (HA). Most germline mutations (>80%) found in *BRCA1* and *BRCA2* result in premature truncated proteins (Friend and Breast Cancer Information Core Steering Committee 1995; Shattuck-Eidens *et al.*1995; Couch *et al.* 1996a; Couch *et al.* 1996b). The protein truncation test (PTT) is therefore often used, especially for the larger exons (exon 11 of *BRCA1* and exon 10 and 11 of *BRCA2*). It is important to note that no single technique currently available can guarantee the identification of all cancer-predisposing mutations in the *BRCA1* and *BRCA2* genes.

Alternative methods are used for detecting large rearrangements, which would not be detected with conventional PCR-based screening techniques. These would include RFLP analysis, Southern blot analysis, or modified PCR-based analyses, e.g. long PCR and real-time PCR.

2.13.1 Single-strand conformation polymorphism / heteroduplex analysis (SSCP/HA)

SSCP analysis and HA are PCR-based mutation scanning technologies. During electrophoresis, single-stranded DNA folds into a three-dimensional shape according to its primary sequence (Orita *et al.* 1989). The electrophoretic mobility of separation then becomes a function of the shape of the folded, single-stranded molecule. The principle of HA, similar to SSCP, depends on the conformation of duplex DNA in a native gel, which consists of complementary strands or strands that have one or more base pair mismatches. Mismatched DNA duplexes (heteroduplexes) have an altered electrophoretic mobility relative to the homoduplexes (Nataraj *et al.* 1999). A number of factors can influence sensitivity in an unpredictable way, such as electrophoretic conditions; including temperature of the gel during electrophoresis, gel composition and the dimensions of the gel. The fragment length and the location of the sequence variation along the DNA fragment should also be considered (Nataraj *et al.* 1999). The addition of glycerol has been empirically shown to enhance mobility shifts, but the reason for this effect has remained unexplained (Hayashi 1999). Kukita *et al.* (1997) proposed that this effect was due to the lowering of the pH of the electrophoresis buffer as a result of the reaction of glycerol and borate.

The simplicity and efficiency of SSCP, as well as its cost-effectiveness (Sevilla *et al.* 2002) makes this mutation-detection method appealing for rapid analysis of large patient groups. Sensitivity is, however, a major cause for concern when using SSCP as a screening method for novel mutations. Analysis of genes other than *BRCA1* or *BRCA2* suggested that only 80% of single base pair substitutions (a higher proportion of small insertions and deletions) can be detected by this technique when performed correctly (Vidal-Puig and Moller 1994; Jordanova *et al.* 1997).

When utilizing SSCP screening of a gene as large as *BRCA1* or *BRCA2*, no one set of conditions or even one method may be optimal for all of the exons. Rather, a variety of different methods and conditions should be studied for each set of amplimers under analysis. One standard protocol or even a single method cannot adequately screen large genes. A few mutations, likely single base pair changes

that tend to produce more subtle gel pattern perturbations, will be missed even in an optimal setting. Detection power is increased when combining the SSCP technique with the HA technique (Kotze *et al.* 1995).

2.13.2 Melt-point analysis by microplate-array diagonal-gel electrophoresis (melt-MADGE)

Melt-MADGE was invented by Day in 1994 (Day and Humphries 1994). It is a PCR-based screening method implementing temporal-thermal-ramp electrophoresis analysing duplex melting. The melt-MADGE system uses the same principle as DDGE or TGGE (thermal gradient gel electrophoresis), but exchanges the dimension of the gradient so that it is a thermal ramp in time rather than a gradient in space. The PCR products for analysis are loaded on a homogeneous gel and the temperature of the entire gel is raised during the course of electrophoresis. It is theoretically 100% sensitive to point and frameshift variations and is backed by a precise mathematical model that predicts the electrophoretic behaviour and resolution of alleles along a temperature gradient (Day *et al.* 1998). This technique allows high-throughput cost-effective screening of large genes.

2.13.3 Direct DNA sequencing (DS)

DS has been proclaimed to be the gold standard for mutation detection (Dianzani *et al.* 1993), although not necessarily 100% accurate as unequal amplification of alleles in heterozygous samples have been reported (Van der Heiden *et al.* 2004). Most other screening techniques, such as SSCP and HA, still require DNA sequencing of detected variants to be able to identify the exact nucleotide change. The most serious disadvantage of direct DNA sequencing is the high-cost involved (Sevilla *et al.* 2002).

In automated DNA sequencing dideoxynucleotides (ddNTPs), labelled with different fluorochromes, are used. The labels are incorporated into the ddNTPs and this is used to carry out chain termination. Alternatively the primers can be labelled with fluorochromes, which mean the same primers cannot be used for the initial PCR amplification and the sequencing reaction. The four chain-terminated products are run on the same track of a denaturing electrophoresis gel. Capillary electrophoresis utilizes liquid polymers in thin capillary tubes. Each product, with their base-specific dye, is excited by a laser and the dye then emits light at its characteristic wavelength. A diffraction grating separates the emission,

which are detected by a charge-coupled device (CCD) and the sequence is interpreted by a computer (Molecular biology and basic techniques 2000).

2.14 OBJECTIVES OF THIS STUDY

The *BRCA1* and *BRCA2* genes have been intensively studied since they were cloned in the mid 1990's. Although allele frequencies of polymorphisms in the *BRCA* genes have been determined and the mutation spectrum identified in various populations across the world, in only one other published study (Reeves *et al.* 2004) *BRCA1* was screened for mutations in South African populations. The breast cancer patients were mostly Caucasians of European descent. In this study we include patients of Mixed Ancestry (consisting of Koi San, Malay, African and European Caucasian ancestry (Nurse *et al.* 1985)), also referred to as the Coloured population. Since *BRCA1* and *BRCA2* are relatively large and also polymorphic genes, more comprehensive studies are needed in all our population groups in order to investigate the implementation of predictive genetic testing for South African families.

The specific aims of this study:

1. Determine the *BRCA1* and *BRCA2* mutation spectrum and prevalence in two populations, Caucasian and Mixed Ancestry, from the Western Cape Province of South Africa.
2. Evaluate the relevance of mutations in these genes in individuals with a moderate breast cancer family history.
3. Determine the frequency of the *CHEK2**1100delC in South African breast cancer patients with a family history (populations described in (1)) and without a family history of breast cancer (Caucasian, Mixed Ancestry, Indian and African patients with sporadic breast cancer).

3. MATERIALS AND METHODS

3.1 PATIENTS

In this study we investigated 101 unrelated patients (98 women and 3 men), presenting with incident invasive breast cancer. Patients were from four South African population groups (Mixed Ancestry n=65, Caucasian n=30, African n=3, Indian n=1 and unknown ethnicity n=2), all accrued from the Tygerberg Academic Hospital in the Western Cape. Participants gave informed consent to the use of their DNA for studying the genetic basis of their breast cancer (refer to Appendix A for an example of the informed consent form the patients signed). Each patient also completed a questionnaire (available in both English and Afrikaans: Appendix B) which included information pertaining to known environmental modifiers of cancer risk, for example a history of smoking, alcohol consumption and hormonal contraceptive use and/or hormone replacement therapy (HRT). Reproductive history was also ascertained for the patients. The study was approved by the Ethics Review Committee of the Faculty of Health Sciences, University of Stellenbosch (2001/C062) and the Medical Research Council of South Africa (2004/07/29).

Patients were divided into two groups according to their family history of cancer. The first group (n=48) is considered to have a modest increased risk for familial breast cancer and was therefore screened for both the *CHEK2**1100delC variant as well as the entire coding sequences of the *BRCA1* and *BRCA2* genes. These patients have previously been screened for the Ashkenazi Jewish founder mutations (*BRCA1* 185delAG and 5382insC and *BRCA2* 6174delT) and the *BRCA1* Afrikaner founder mutation (E881X) in another study by C Scholtz and MJ Kotze. The familial group included individuals from the Caucasian (n=19) and Mixed Ancestry (n=29) population groups. Only one case of bilateral breast cancer was observed in this group (first cancer at 69 years of age in right breast, second cancer at 77 years of age in left breast and bilateral breast cancer at the age of 83 years). The age distribution of this group is summarized in Table 3.1. Fifty-six percent of the patients were diagnosed between the ages of 50 and 70 years. Table 3.2 contains the family history of cancer in these patients. An excess of stomach, oesophageal, colon, pancreatic and prostate cancer was noted in the families of this patient group. Only two patients reported family members with ovarian cancer.

Table 3.1 Age of onset distribution for the familial breast cancer patient group.

Population	**Age Distribution**						
	31-40 years	**41-50 years**	**51-60 years**	**61-70 years**	**71-80 years**	**81+ years**	**Total**
Mixed Ancestry	8	5	8	5	2	1	29
Caucasian	0	1	8	6	4	0	19
Total	**8**	**6**	**16**	**11**	**6**	**1**	**48**

Table 3.2 Family history of cancer for the familial breast cancer group. Patients have been divided into two groups according to age of onset of breast cancer.

Proband	**Family history of 1st and 2nd degree relatives**							
	BC only	**BC and/or stomach cancer**	**BC and/or oesophageal cancer**	**BC and/or ovarian cancer**	**BC and/or colon cancer**	**BC and/or pancreatic cancer**	**BC and/or prostate cancer**	**Other cancers**
Breast cancer ≤50 years	2	3	2	0	0	0	1	6
Breast cancer >50 years	15	3	1	2	3	3	2	5
Total	**17**	**6**	**3**	**2**	**3**	**3**	**3**	**11**

BC - breast cancer

The second group (n=53: Mixed Ancestry n=36, Caucasian n=11, African n=3, Indian n=1 and unknown ethnicity n=2) is regarded as sporadic cases and was therefore only screened for the *CHEK2**1100delC variant. The sporadic group included only one male patient of Mixed Ancestry. Of these patients, 44 (83%) reported not to have a family history of any type of cancer. Four patients (0.08%) reported one family member previously diagnosed with breast cancer. The age distribution of this group is summarized in Table 3.3. Fifty-three percent of the patients were diagnosed between the ages of 50 and 70 years. Although the prevalence of the mutation has been reported to be greater in women with a family history of breast cancer (Meijers-Heijboer *et al.* 2002; Vahteristo *et al.* 2002), an increased breast cancer risk has also been shown to be evident in women unselected for a family history (The CHEK2 Breast Cancer Case-Control Consortium 2004).

Table 3.3 Age of onset distribution for the sporadic breast cancer patient group.

Population	**Age Distribution**					
	31-40 years	**41-50 years**	**51-60 years**	**61-70 years**	**71-80 years**	**Total**
Mixed Ancestry	3	8	12	6	7	36
Caucasian	1	0	4	3	3	11
African	0	1	1	0	1	3
Indian	0	0	1	0	0	1
Ethnicity unknown	0	1	1	0	0	2
Total	**4**	**10**	**19**	**9**	**11**	**53**

3.2 METHODS

3.2.1 DNA extraction

Genomic DNA was extracted from whole venous blood according to an adapted salting out procedure (Miller *et al.* 1988).

Blood was collected in 5ml EDTA (vacutainer) tubes. These tubes were emptied into 40ml Greiner tubes (Greiner Labortechnik) and cold lysis buffer (0.155M NH_4Cl; 0.01M $KHCO_3$; 0.0001M EDTA; pH7.4) was added to a final volume of 40ml. The mixture was kept on ice for 30 minutes and inverted every 5 minutes. A 45 minutes centrifugation step at 350rcf (relative centrifugal force) at 4°C followed. Subsequently the supernatant was discarded and the pellet washed with 20ml PBS (phosphate buffered saline) (0.027M KCl; 0.137M NaCl; 0.008M Na_2HPO_4; 0.0015M KH_2PO_4). This solution was centrifuged for 30 minutes at 350rcf at 4°C, after which the supernatant was discarded and the pellet resuspended in 3ml nucleic lysis buffer (0.01M Tris-HCl; 0.4M NaCl; 0.002M EDTA; pH8.2), 60µl 10mg/ml proteinase K and 300µl 10% (w/v) SDS. After an overnight incubation period at 55°C, 2ml of a saturated NaCl solution was added and the tube shaken vigorously for 1 minute. The tube was then centrifuged for 30 minutes at 1000rcf at 21°C. Subsequently the supernatant was transferred to a new Greiner tube, leaving the foam and pellet behind. The supernatant was shaken for another 15 seconds and centrifuged for 15 minutes at

500rcf at 21°C. The supernatant was transferred to a third Greiner tube. Two volumes of cold 100% (v/v) ethanol were added to precipitate the DNA. The gDNA spindle which formed was transferred with a sterile plastic inoculation needle to a clean 1.5ml Eppendorf tube containing 1ml 70% (v/v) ethanol. The tube was centrifuged at 17500rcf for 10 minutes at 4°C. The ethanol was poured off and the pellet left at room temperature to dry. Depending on the size of the pellet, 100-500µl SABAX water was added to resuspend the DNA. To ensure a homogenous solution, samples were stored for at least 24 hours at 4°C before being subjected to analysis.

3.2.2 DNA quantification

DNA concentration and purity were determined using the NanoDrop® ND-100 Spectrophotometer v3.0.1 (NanoDrop Technologies Inc, DE, USA) or with gel electrophoresis, using lambda DNA as standards. Dilutions were made where possible to obtain a final concentration of not less than 30ng/µl.

3.2.3 Polymerase chain reaction (PCR) primer design

3.2.3.1 *BRCA1*

Several of the *BRCA1* exons have been screened previously in the study population (Appendix C, Scholtz *et al.* 2003). These include exons 2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16 and exon 20. The primer design and screening protocol for these fragments will not be discussed. Primers were designed to screen the remaining coding exons of the *BRCA1* gene (exons 11, 15, 17, 18, 19, 21, 22, 23 and exon 24) using the GenBank reference sequences U14680. The analysis excluded exons 1 and 4, since exon 1 is non-coding and exon 4 is only a splicing variant. Because of its size, exon 11 was screened using 14 overlapping primer sets, including one set designed to screen for the E881X mutation. Primers were placed ≥40 nucleotides away from the 5' splice boundary of each exon, and ≥15 nucleotides away from the 3' splice boundary in order to ensure thorough screening of all splice sites. By keeping the fragments relatively small, the sensitivity of the screening technique, namely SSCP/HA, was maximised. The final list of primer sequences appear in Table 3.4.

3.2.3.2 *BRCA2*

Primers were designed to screen the 26 coding exons of the *BRCA2* gene, using the GenBank reference sequence U43746 and the on-line primer design software, Primer3 (available at www.http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The same criteria were used as for the design of the *BRCA1* primers. Exon 11 of *BRCA2* is even larger than that of *BRCA1* (~5kb *versus* ~3kb). It was therefore considered to be more cost-effective and less time consuming to screen exon 11 by means of direct DNA sequencing. The exon was amplified in 7 fragments and sequenced in both directions. Exon 10 is also relatively large and had to be divided into 5 fragments to allow for SSCP/HA. Both exons 10 and 11 primers were designed to allow ample overlap within the exons. Primer sequences are listed in Table 3.5.

3.2.3.3 *CHEK2*

A number of regions of the human genome exhibit a high homology to exons 10 to 14 of *CHEK2*, making mutational analysis problematic (Sodha *et al.* 2000). The *CHEK2**1100delC falls within exon 10. The primer set which was used for the detection of this variant could discriminate between the functional gene (GenBank AL131197) and homologous copies of the fragment in the pseudogenes (forward primer 5'GCA AGT TCA ACA TTA TTC CCT TTT 3'; reverse primer 5'ATC ACC TCC TAC CAG TCT GTG C 3') (Sodha *et al.* 2002a). Amplification yielded a 245bp amplicon, suitable for single strand conformation polymorphism and heteroduplex analysis (SSCP/HA).

3.2.4 Polymerase chain reaction (PCR) amplification

PCR was carried out in 25µl volumes containing ~30ng of genomic DNA, 1X NH_4 PCR buffer (supplied with the DNA polymerase), 0.1mM each of dATP, dGTP, dTTP and dCTP (Promega Corporation, Madison, WI, USA), 1.5mM $MgCl_2$ and 0.5U *Taq* polymerase (BIOTAQ^{TM} DNA polymerase, Bioline Ltd., London, England). The PCR conditions were individually optimized by varying the primer concentrations, which are listed in Tables 3.4 and 3.5 for *BRCA1* and *BRCA2* respectively.

Three PCR cycling regimes were used for amplifying *BRCA1* and *BRCA2* fragments. The first program (Program A) comprised an initial denaturation step at 94° for 5 minutes, 35-40 cycles

consisting of denaturation at 94°C for 30 seconds, annealing (see Tables 3.4 and 3.5 for temperatures) for 45 seconds and extension at 72°C for 30 seconds with a final extension period at 72°C for 5 minutes. The second program (Program B) comprised an initial denaturation step at 95°C for 5 minutes, 35 cycles consisting of denaturation at 95°C for 1 minute and annealing (see Tables 3.4 and 3.5 for temperatures) for 2 minutes. A terminal 10 minute extension at 72°C completed the amplification. The third program is a two-step cycling regime (Program C). The regime comprised an initial denaturation step at 95°C for 2 minutes, 40 cycles with a 95°C denaturation and a 72°C elongation step (both for 30 seconds), for which the first 10 cycles the annealing temperature was higher than the annealing temperature for the last 30 cycles (for 45 seconds in both instances). The program was completed with a final elongation step at 72°C for 2 minutes. Various PCR cycling regimes were tested to amplify exon 10 of the *CHEK2* gene, including Program A and Program C at different annealing temperatures. Another regime (Program D) was tested. This is a touch-down program with the denaturation and extension similar to program C. During the first cycle the annealing temperature is between 60°C and 65°C, decreasing by 0.5°C after each cycle, for 10 cycles. This is followed with 30 cycles at the annealing temperature of the last touch-down cycle. The PCR thermocyclers used were the Perkin Elmer GeneAmp PCR System, models 9700 and 2700 (Perkin Elmer Applied Biosystems, Warrington WA, Great Britain).

Three of the *BRCA2* exons were amplified by means of a nested-PCR. Exon 15 was amplified using the forward primer for exon 14B and the reverse primer for exon 15 (10pmol/µl of each primer; Program B; 66°C annealing temperature). Exon 20 was amplified using the forward primer for exon 19 and the reverse primer for exon 20 (5pmol/µl of each primer; Program B; 55°C annealing temperature). Exon 22 was amplified using the forward primer for exon 22 and the reverse primer for exon 23 (5pmol/µl of each primer; Program B; 60°C annealing temperature). One microlitre of the amplification product was subsequently used as the template for the nested reaction in each instance. The conditions for these reactions can be found in Table 3.5.

The intensity and size of the PCR products were determined on a 1.5% agarose gel with 1X TBE running buffer (stock 5X TBE: 0.089M Tris; 0.089M boric acid; 0.002M EDTA), with ethidium bromide incorporated in the gel at a concentration of 0.5µg/ml. A 100bp DNA molecular marker (Promega Corporation, Madison, WI, USA) was loaded alongside the amplicons. Electrophoresis was performed at 120V for at least 40 minutes. The fragments were visualised with UV light and captured using the MultiGenius Image Capture System (Syngene, Cambridge, England).

Table 3.4 Primer sequences and conditions for amplifying and analysing the various *BRCA1* fragments. Primer sequences are cited in the 5'→3' direction.

Exon	Forward Primer	Reverse Primer	Primer Concentration (pmol/reaction)	PCR Program	Annealing Temp	Amplicon Size (bp)	Mutation Detection
11A	TAGCCAGTTGGTTGATTTCC	CCCATCTGTTATGTTGGCTC	15	C	60/55°C	394	SSCP/HA
11B	CCATGTGGCACAAATACTCA	TGATTCAGACTCCCCATCAT	15	C	60/55°C	399	SSCP/HA
11C	GAAACTGCCATGCTCAGAGA	ATTTATTTGTGAGGGGACGC	15	C	60/55°C	437	SSCP/HA
11D	AGGAGCATTTGTTACTGAGC	TTTAATTCGAGTTCCATATTGC	15	A	60°C	348	SSCP/HA
11E	TTCAAAACGAAAGCTGAACC	TTGGAAGGCTAGGATTGACA	15	C	60/55°C	445	SSCP/HA
11F	GGTAAAGAACCTGCAACTGG	TCAAATGCTGCACACTGACT	15	C	65/60°C	416	SSCP/HA
11G	GAAAGGGTTTTGCAAACTGA	TTCCTCTTCTGCATTTCCTG	15	C	60/55°C	381	SSCP/HA
11H	TGAACTTGATGCTCAGTATTTGC	AGTCCAGTTTCGTTGCCTCT	15	B	60°C	345	SSCP/HA
11I	TAAGCCAGTTGATAATGCCA	TTTTGGCCCTCTGTTTCTAC	15	C	65/60°C	430	SSCP/HA
11J	ACTAATGAAGTGGGCTCCAG	TAACCCTGAGCCAAATGTGTAT	15	A	66°C	455	SSCP/HA
11K	TTCCTGGAAGTAATTGTAAGCA	TAACCCTGAGCCAAATGTGTAT	15	B	60°C	313	SSCP/HA
11L	GACATTAAGGAAAGTTCTGCTG	TTTGCCAATATTACCTGGTTAC	15	A	60/55°C	329	SSCP/HA
11M	TGTCTAAGAACACAGAGGAG	GGGGCAAACACAAAAACCTG	15	A	60°C	374	SSCP/HA
E881X	GTCGGGAAACAAGCATAGAAAT	TGATATTAACTGTCTGTACAGG	30	C	62/55°C	258	SSCP/HA
15	TTGCCAGTCATTTCTGATCT	GTGTTTGTTCCAATACAGCAG	15	C	60/55°C	347	SSCP/HA
17	GTGCAGGATTGCTACATAGG	CAAAGTGCTGCGATTACAGG	15	B	60°C	290	SSCP/HA
18	AGTGGTGTTTTCAGCCTCTG	CTCAGACTCAGCATCAGCAA	15	C	63/60°C	342	SSCP/HA
19	TTAAAGGGCTGTGGCTTTAG	AAGGAAAGTGGTGCATTGAT	15	C	63/60°C	279	SSCP/HA
21	CAGGTGGTGAACAGAAGAAA	ACATTTCAGCAATCTGAGGA	15	C	65/60°C	298	SSCP/HA
22	CATCCGGAGAGTGTAGGGTA	CATCCATAGGGACTGACAGG	15	C	65/60°C	240	SSCP/HA
23	CCCTGTCTCAAAAACAAACA	CAAGCACCAGGTAATGAGTG	15	C	60/55°C	234	SSCP/HA
24	TGGAGTCGATTGATTAGAGC	AGCCAGGACAGTAGAAGGAC	15	C	65/60°C	311	SSCP/HA

Temp – temperature; SSCP/HA – single-strand conformation polymorphism / heteroduplex analysis

Table 3.5 Primer sequences and conditions for amplifying and analysing the various *BRCA2* fragments. Primer sequences are cited in the 5'→3' direction.

Exon	Forward Primer	Reverse Primer	Primer Concentration (pmol/reaction)	PCR Program	Annealing Temp	Amplicon Size (bp)	Mutation Detection
2	TTCCCATCCTCACAGTAAGC	GTACTGGGTTTTTAGCAAGC	30	B	66°C	334	SSCP/HA
3	TCTTTAACTGTTCTGGGTCAC	GTTTGTAGTTCTCCCCAGTC	15	B	60°C	360	SSCP/HA
4	CTCCCTATACATTCTCATTCC	TCTTCTACCAGGCTCTTAGC	15	B	60°C	345	SSCP/HA
5&6	CAACAATTTATATGAATGAGAATC	CAAAGGTATAACGCTATTGTC	15	B	60°C	352	SSCP/HA
7	AGCATTCTGCCTCATACAGG	ACAATTATCAACCTCATCTGC	15	B	60°C	291	SSCP/HA
8	CACTGTGTTGATTGACCTTTC	AAGGCATTCCAAAATTGTTAGC	15	B	60°C	279	SSCP/HA
9	TGAAATCACCAAAAGTGAAACC	GGTGACAGAGCAAGACTCC	15	B	60°C	300	SSCP/HA
10A	TTTCTATGAGAAAGGTTGTGAG	TGAGTATTTTTCTTTCACTTGG	10	B	60°C	348	SSCP/HA
10B	TCATTATGTTTTTCTAAATGTAGA	TCTGAAATATTTTGGTCACATG	10	B	55°C	350	SSCP/HA
10C	GTCTTTGGCCTGTGAATGG	ATATAGACTTTTTGATACCCTG	10	B	60°C	350	SSCP/HA
10D	GTAAATAAGAGAGATGAAGAGC	TTAAACCTGCATTCTTCAAAGC	10	B	60°C	346	SSCP/HA
10E	CTGTTTGCTCACAGAAGGAG	AAAAACACAGAAGGAATCGTCA	10	B	60°C	349	SSCP/HA
11A	ACTGTGCCCAAACACTACC	CTTCAGAGTCTGGATTGACAG	10	B	66°C	829	DS
11B	GTTGAGCTGTTGCCACCTG	CAAACTGACTTCCTGATTCTTC	10	B	63°C	886	DS
11C	CATTTACAGAGTAGTGTAGTTG	CTGATCAGTAAATAGCAAGTCC	10	B	60°C	873	DS
11D	CAAGAGAAATACTGAAAATGAAG	TCTGCATTTCTTTACACTTTGG	10	B	58°C	848	DS
11E	TTTTGATGAAAAAGAGCAAGG	AATTATTACTATTAGATATGGAC	10	B	56°C	875	DS
11F	CTAGTTTTTCCAAAGTAATATCC	GTGTAAGGAACTTTCTAAAATGG	10	B	60°C	869	DS
11G	TCACAAGAGAAGAAAATACTGC	CACTCTTAATTGTTAGCATACC	10	B	63°C	860	DS
12	CATTTAAAGAGTCAATACTTTAGCT	GCACAGTGGCTCATGTCTGTA	10	B	60°C	329	SSCP/HA
13	GAGCATCTGTTACATTCACTG	AAACGAGACTTTTCTCATACTG	15	B	60°C	253	SSCP/HA
14A	TGTAGCAAATGAGGGTCTGC	TTTGGTTGGTCTGCCTGTAG	15	B	66°C	296	SSCP/HA
14B	AAGCAATTTAGCAGTTTCAGG	ACGGAAATATCTAACTGAAAGG	15	B	60°C	375	SSCP/HA

Temp – temperature; DS – direct sequencing; SSCP/HA – single-strand conformation polymorphism / heteroduplex analysis

Table 3.5 Continues from previous page.

Exon	Forward Primer	Reverse Primer	Primer Concentration (pmol/reaction)	PCR Program	Annealing Temp	Amplicon Size (bp)	Mutation Detection
15*	CAAAGTGCTGGGATTACAGG	ACACTCTGTCATAAAAGCCATC	10	B	66°C	325	SSCP/HA
16	AATTTTTGGTAAATTCAGTTTGG	GAGAAGAAAGAGGGATGAGG	10	B	55°C	354	SSCP/HA
17	GATCTTGAACAATGTAGTTTTTG	CAGAAACCTTAACCATACTGC	15	B	60°C	331	SSCP/HA
18A	TTCTCAGTTATTCAGTGACTTG	TTCAATAATGGCCACTTTTTGG	15	B	60°C	268	SSCP/HA
18B	ATGGAAAGGGATGACACAGC	GAATTTAACTGAATCAATGACTG	15	B	60°C	358	SSCP/HA
19	GCAGTTCTAGAAGAATGAAAAC	CAAGAGACCGAAACTCCATC	15	B	60°C	339	SSCP/HA
20*	ATTACAGATGTGAGCCACTG	GTTAAATTCAAAGTCTCAAGAC	10	B	66°C	309	SSCP/HA
21	TCTTTAAATCTCCCTTCTTTGG	GCCAGAGAGTCTAAAACAGC	30	B	60°C	297	SSCP/HA
22*	GTTGTATTTGTCCTGTTTAAAGC	TTAATAAAACTGATAAAAACAAAGC	10	B	60°C	331	SSCP/HA
23	AAGCAAAATCCACTACTAATGC	GAGATTCCATAAACTAACAAGC	15	B	60°C	349	SSCP/HA
24	AACAACTACCGGTACAAACC	CAAATTTGCCAACTGGTAGC	15	B	60°C	343	SSCP/HA
25	ATATTAGAGTTTCCTTTCTTGC	GACTGTCAAAATAGAAAAATACC	15	B	60°C	384	SSCP/HA
26	ACAATTGGTATCACATTTAGGG	GATGGCCTCCATATATACTTC	15	B	60°C	338	SSCP/HA
27A	AGGGGAGGGAGACTGTGTG	AATGGGACTAACAGGTGGAG	15	B	60°C	331	SSCP/HA
27B	ATTGATGACCAAAAGAACTGC	TGTTGAACCAGACAAAAGAGC	30	B	60°C	297	SSCP/HA
27C	AATTCTCCTCAGATGACTCC	AACTGGAAAGGTTAAGCGTCA	15	B	60°C	354	SSCP/HA

Temp – temperature; DS – direct sequencing; SSCP/HA – single-strand conformation polymorphism / heteroduplex analysis

* Exons amplified by means of a nested-PCR. See text for PCR conditions for the first amplification on gDNA.

3.2.5 Single strand conformation polymorphism and heteroduplex analysis (SSCP/HA)

SSCP/HA was used for detecting the *CHEK2**1100delC variant and to screen *BRCA1* and *BRCA2* (excluding exon 11) for known and novel variants.

Denaturing loading buffer (95% (v/v) formamide, 10mM EDTA pH8.0, 0.05% (w/v) xylene cyanole and 0.05% (w/v) bromophenol blue) were added to the amplified DNA fragments in a 1:1 ratio. The samples were than denatured for 10 minutes at 95°C, cooled rapidly to 4°C and kept on ice until it could be loaded onto the gel. This was done to allow single-strand conformations and homo- and heteroduplexes to form. Of the sample and buffer solution 10-20µl (depending on the PCR product yield after amplification) were loaded on a 12% (w/v) polyacrylamide gel (99 acrylamide : 1 bisacrylamide, 1% cross linking) supplemented with 7.5% (w/v) urea. The dimensions of the gel system (Duel Slab Gel Unit, C.B.S. Scientific Co., Del Mar, CA, USA) used were 165mm (width) x 370mm (height) x 0.75mm (depth). The mixture for one 50ml gel consisted of 15ml of 40% (w/v) polyacrylamide stock, 15 ml of 5X TBE, 20 ml dH_20, 800µl of 10% (w/v) ammonium persulphate (APS) and 80µl of TEMED. 1.5X TBE was used as the running buffer. Electrophoresis was performed at 4°C for 14-20 hours (depending on the fragment size) at 350V. The banding patterns were subsequently visualized by either ethidium bromide staining or by silver staining.

All fragments were analysed according to the protocol described above, except for exon 9 of *BRCA2*. In an attempt to improve the resolution of the SSCP banding pattern for this fragment, different gel matrices were tested. This included a 10% (w/v) polyacrylamide gel (99 acrylamide : 1 bisacrylamide, 1% cross linking) supplemented with 0.05% glycerol, for which a 0.5X TBE running buffer was used and electrophoresis took place at room temperature at 200V. The other gel matrix tested was a 20% (w/v) polyacrylamide gel (99 acrylamide : 1 bisacrylamide, 1% cross linking) without glycerol or urea and 0.5X TBE as the running buffer. Electrophoresis was performed at 4°C at 200V for at least 20 hours.

One of the exon 11 fragments (11J) of the *BRCA1* gene is relatively large, 455bp, which could compromise the sensitivity of the screening method. The fragment was therefore digested with a restriction enzyme in order to reduce the size of the amplicon. *MvaI* (Roche Diagnostics Corporation, Indianapolis, IN, USA) was used which cuts the fragment only once, in a 1:2 ration, yielding a 146bp and a 309bp product.

In addition to the system described above, the *CHEK2* fragment was also analysed on a Mighty Small (Hoefer Pharmacia Biotech Inc, California, USA) system. The dimensions of the gel used were 100mm (width) x 105mm (height) x 0.75mm (depth). A 10% as well as a 15% (w/v) polyacrylamide gel (99 acrylamide : 1 bisacrylamide, 1% cross linking) were tested. Electrophoresis was done in the presence of 1.5X TBE and 1X TBE running buffer respectively, at room temperature at 200V for 2 hours.

3.2.6 Silver staining

Gels were loosened from the glass plates and subjected to a 10 minute incubation period in fixing solution (10% (v/v) ethanol, 0.5% (v/v) acetic acid) after which each gel was rinsed for approximately 1 minute in dH_2O. Another 10 minute incubation period with staining solution (0.1% (w/v) silver nitrate) followed. After staining, the gels were briefly rinsed with dH_2O. Finally the gels were developed with developing solution (1.5% (w/v) NaOH and 0.155% (v/v) formaldehyde which was only added when staining commenced). The last step continued until clear bands could be seen. Developing ended after the gels were rinsed with dH_2O and sealed between transparencies. All incubation steps were performed on a belly-dancer (Stoball Life Sciences Inc, NC, USA) set at low speed.

3.2.7 Melt-point analysis by microplate-array diagonal-gel electrophoresis (melt-MADGE)

In addition to SSCP/HA, exon 11 of the *BRCA1* gene was screened with melt-MADGE by microplate-array diagonal-gel electrophoresis) by INM Day. This allowed comparison of the sensitivity of the two techniques. Samples with reported variations were subsequently sequenced (refer to next section).

3.2.8 Sequence analysis

All the samples were subjected to SSCP/HA analysis for the *BRCA1* exons and *BRCA2* exons (with the exception of exon 11). Only when aberrant mobility was detected on the SSCP/HA gels, the samples which showed variation were analysed by DNA sequence analysis. The samples were re-amplified and purified with the Qiaquick PCR purification kit (Qiagen, GmbH, Hilden, Germany) or with the GFX PCR DNA and gel band purification kit (Amersham Biosciences Corporation, Buckinghamshire, England) according to manufacturers' instructions. The concentrations of the

amplification products were subsequently determined with either the NanoDrop® ND-100 Spectrophotometer v3.0.1 (NanoDrop Technologies Inc, DE, USA) or with gel electrophoresis using lambda DNA as concentration standards. Samples were then diluted to a final concentration of 3.3ng/µl. Five samples were randomly chosen for DNA sequencing of the *CHEK2* fragment in order to confirm amplification of the correct sequence. These samples were prepared for sequencing according to the protocol described above. *BRCA2* exon 11 fragments were amplified and the concentrations determined using gel electrophoresis and lambda concentration standards. Samples were diluted to a final concentration of 6.6ng/µl and subsequently purified with ExoSAP-IT (Amersham Biosciences Corporation, Ohio, USA).

The PCR products of the samples were sequenced bidirectional (5' to 3' and 3' to 5'), using an ABI PRISM di-deoxy Terminator Cycle Sequencing kit v3.1 and the ABI Prism 3100 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Warrington WA, Great Britain) according to manufacturers' instructions. Electropherograms of each amplicon were analyzed by proprietary sequence analysis software followed by visual inspection and confirmation, assisted by sequence alignments with the wild-type reference sequence for each gene. Sequence alignments were done using the BioEdit Sequence Alignment Editor v6.0.7 (Hall 1999).

BRCA1 and BRCA2 amino acid conservation between species were determined for the detected unclassified variants which did cause an amino acid change in the peptide. Alignments were done using the canine (*Canis familiaris*) homolog with GenBank accession NM001013416 for *BRCA1* and NM001006653 for *BRCA2*. GenBank accession NM009764 was used for the murine (*Mus musculus*) *BRCA1* homolog and NM009765 for the *BRCA2* homolog. Analyses were done using the BioEdit Sequence Alignment Editor v6.0.7 (Hall 1999).

The presence of unclassified *BRCA1* and *BRCA2* variants were established in ethnically-matched control samples. Genotyping was only done for novel variants which caused non-conservative amino acid changes or inframe deletions or insertions. The control samples comprised 50 random individuals of the appropriate South African population group.

3.2.9 Nucleotide numbering

3.2.9.1 *BRCA1* and *BRCA2*

In the interest of consistency, nucleotides were numbered according to the Breast Cancer Information Core (BIC) mutation database (http://research.nhgri.nih.gov/bic/) (Szabo *et al.* 2000). The BIC database has adopted most of the nomenclature system recommendations from Den Dunnen and Antonarakis (2001) for designation of mutations in the *BRCA1* and *BRCA2* genes as derived from the cDNA GenBank reference sequences U14680 and U43746, respectively. One significant departure from these recommendations is that the cDNA number system used by the BIC designates the first nucleotide of the GenBank entry as nucleotide #1. For *BRCA1*, this entry includes 119 bases of 5' untranslated sequence with protein translation starting at nucleotide #120. The reference *BRCA2* sequence contains a 5' untranslated region of 228 nucleotides. Although the untranslated segments must be taken into account when calculating the nucleotide numbers, it should not be included when converting from the cDNA position to the codon number (codon one being the first ATG after the transcription start site).

3.2.9.2 *CHEK2*

The *CHEK2* gene sequence is numbered according to Den Dunnen and Antonarakis (2001) with GenBank accession AL131197 used as the wild type sequence.

3.2.10 Statistical analysis

Allele and genotype frequencies were only calculated for variants which appeared to be relatively common in the study population of breast cancer patients. Hardy-Weinberg equilibrium (HWE) was tested for one detected *BRCA2* variant for which possible *in utero* selection has been reported (Healey *et al.* 2000) (refer to section 5.2.2.1 for a detailed discussion). This was done using Fisher's exact test. This test statistic is more appropriate to use for smaller sample sizes than chi-square analysis (Elston and Forthofer 1977). The computer software Tools for Population Genetic Analysis, TFPGA (Miller and Mark 1997) was used to calculate the test statistic and *P* values.

4. RESULTS

The *BRCA1* and *BRCA2* variants detected in the two South African breast cancer populations will be presented in this chapter. The outcome of the *CHEK2**1100delC screening will also be addressed. Results reported in this chapter have been presented at several national conferences (Appendix C) and will be submitted for publication shortly.

4.1 THE *BRCA1* GENE

The study population was previously screened for the *BRCA1* Ashkenazi Jewish founder mutations 185delAG and 5382insC and the Afrikaner founder mutation (E881X) (see Appendix C, Scholtz *et al.* 2003). The 185delAG and the 5382insC mutations were not detected in the study population. The E881X mutation was identified in one individual (refer to Table 4.1).

4.1.1 Polymerase chain reaction (PCR) amplification

The coding exons of the *BRCA1* gene were amplified in 22 fragments, excluding the exons (2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16 and) that have been screened previously as part of another study (Appendix C, Scholtz *et al.* 2003). The 22 fragments include the 14 fragments which comprise exon 11, of which one has been designed to specifically screen for the E881X mutation. A second primer set had to be designed for exon 11D since the first pair could not be optimized. A second forward primer was designed for exon 17 for the same reason. All amplicons were resolved on a 1.5% agarose gel to confirm successful amplification before SSCP/HA was conducted. A representative example of an agarose gel showing the overlapping fragments of exon 11 are presented in Figure 4.1. Fragment sizes can be derived from the 100bp DNA molecular marker loaded in the first lane. PCR amplification and band size verification were done for all the other fragments and compared with the fragment length calculated from the reference sequence in order to confirm amplification of the correct fragment. A negative control (i.e. without template DNA) was always included to exclude possible PCR contamination.

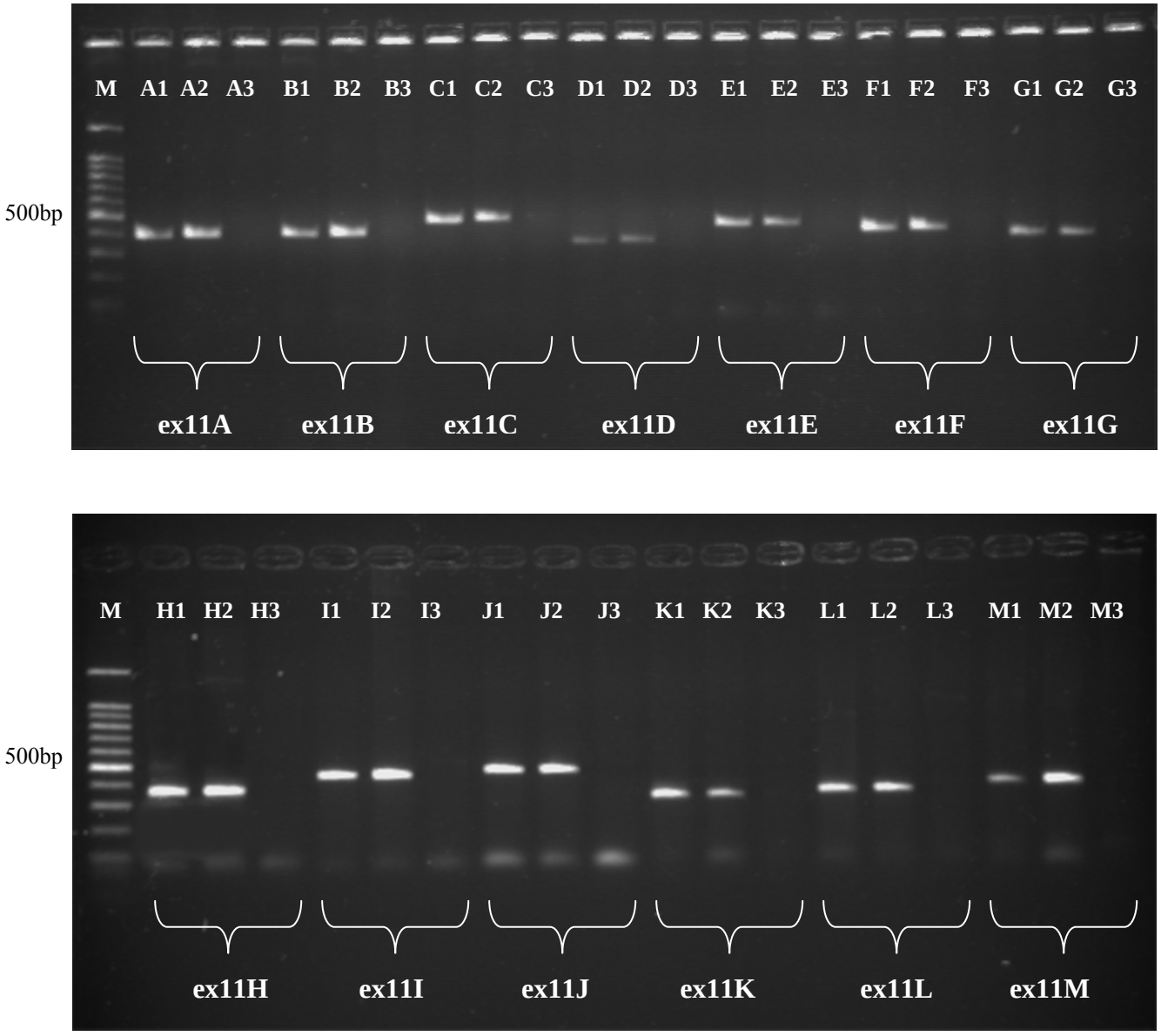

Figure 4.1 *BRCA1* exon 11 fragments amplified by PCR. The two 1.5% agarose gels were resolved for 1 hour at 100V in 1X TBE buffer and stained with 0.5µg/µl EtBr. The photographs were taken after exposure to UV light. 100bp molecular markers are loaded in the first lanes (M) of each gel, with the 500bp band indicated. Lanes A1 to M2 represent the different fragments for exon 11, with the negative control loaded in the third lane of each fragment (A3 to M3).

Exon 11 fragments were screened with SSCP/HA, a screening method which does not require high PCR product concentrations. The staining method used (silver staining and ethidium bromide staining) can also be adapted to increase band visibility on polyacrylamide gels. Lower concentrations of PCR

products, for examples exon 11D in Figure 4.1, would therefore not hamper the screening process. Primer dimers were still present for some of the fragments (for example exon 11J and 11M) after extensive attempts at optimization of the PCR reactions. Since SSCP/HA was employed, the short dimer fragments (~20bp) did not interfere with the banding patterns of the fragments on the polyacrylamide gels, as it moved from the gel into the running buffer after a period of 16 hours. Neither the low concentrations of PCR amplification products or the presence of primer dimers were a concern for DNA sequencing of the variant bands. The fragments are relatively small (<450bp), requiring only 3.3ng/sequencing reaction. All PCR products were purified with either the Qiaquick PCR purification kit or the GFX PCR DNA purification kit before sequencing (refer to section 3.2.8). Both of these commercial products separate the amplified products from primer dimers.

4.1.2 Single strand conformation polymorphism / heteroduplex analysis (SSCP/HA)

Only one SSCP gel electrophoresis system was used to screen the *BRCA1* gene for sequence variations. All fragments were resolved on a 12% (w/v) polyacrylamide gel supplemented with 7.5% (w/v) urea. This system allowed for clear banding patterns which could unambiguously be identified. All fragments were electrophoresed overnight (minimum 16 hours) except for exon 11B which was electrophoresed for only 4 hours to improve the resolution. Exon 11J was digested with the restriction enzyme *MvaI*, yielding a 146bp and a 309bp product. Although faint, banding patterns for both fragments could be distinguished on the SSCP/HA gel. No variants were detected. The original large fragment was also subjected to SSCP/HA analysis to confirm the results.

SSCP/HA gels from four of the exon 11 fragments with variant migration patterns are shown in Figures 4.2 to 4.5. Exon 11 fragments overlapped. Some of the variants could therefore be detected with more than one primer set (e.g. P871L in the fragments amplified with the E881X primer set and with the exon 11G primer set. See Figures 4.2 and 4.3). The variants have been characterized by subsequent sequencing of these samples. Single base substitutions were mostly observed with SSCP analysis. Small deletions manifested with HA (e.g. Figure 4.5). Relatively few of the detected variants changed the conformation of both the single-stranded DNA and the heteroduplexes (Figure 4.4 shows one of these exceptions).

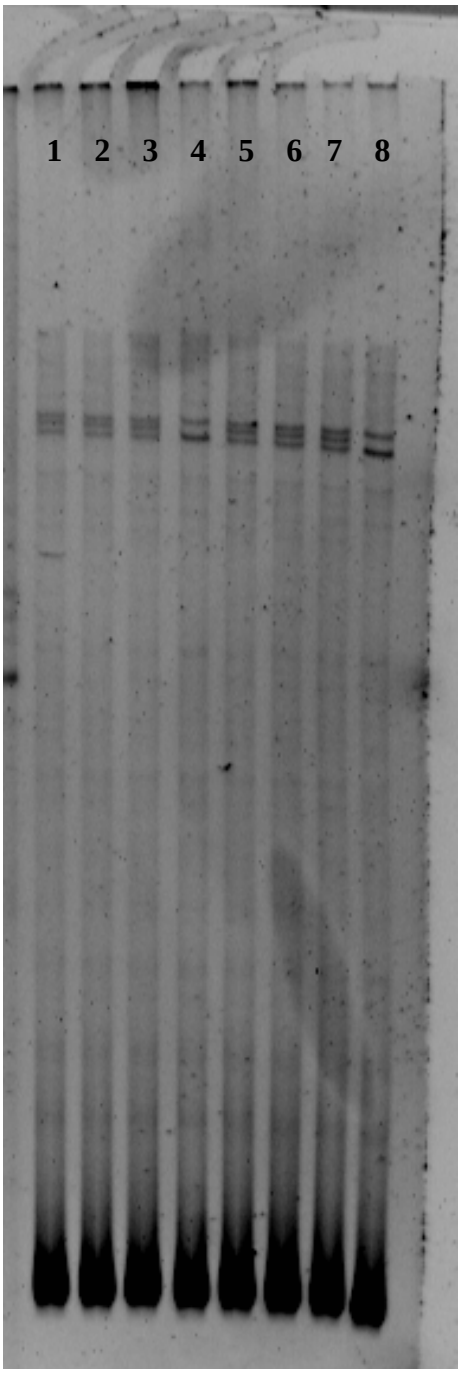

Figure 4.2 *BRCA1* exon 11G fragment. SSCP/HA polyacrylamide gel stained with EtBr. Individuals with both the L771L and the P871L variant can be seen in lanes 1-3 and lanes 5-7. Individuals with only the P871L variant appear in lanes 4 and 8.

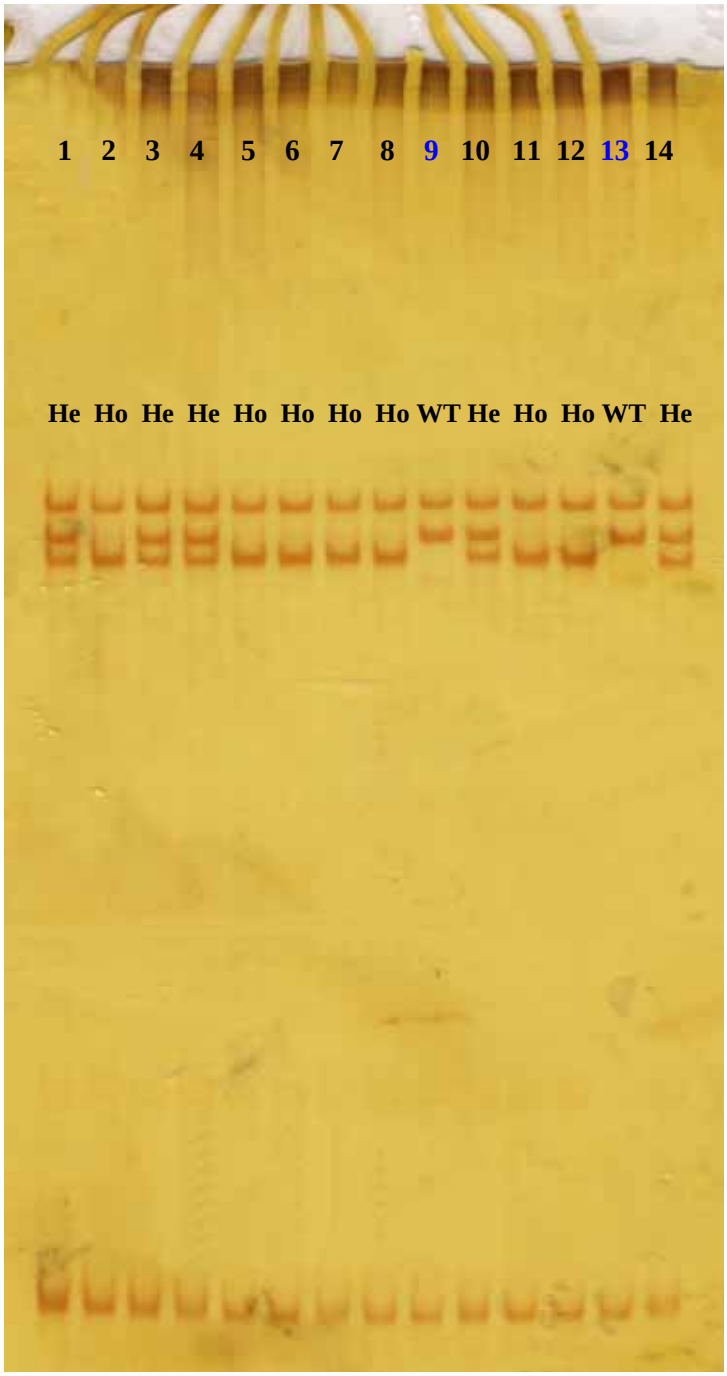

Figure 4.3 *BRCA1* exon 11 fragment using the E881X primers for PCR amplification. SSCP/HA polyacrylamide gel stained with $AgNO_3$. Heterozygous (He) and homozygous (Ho) individuals for the P871L variant are indicated, as well as the individuals with the wild-type sequence (WT).

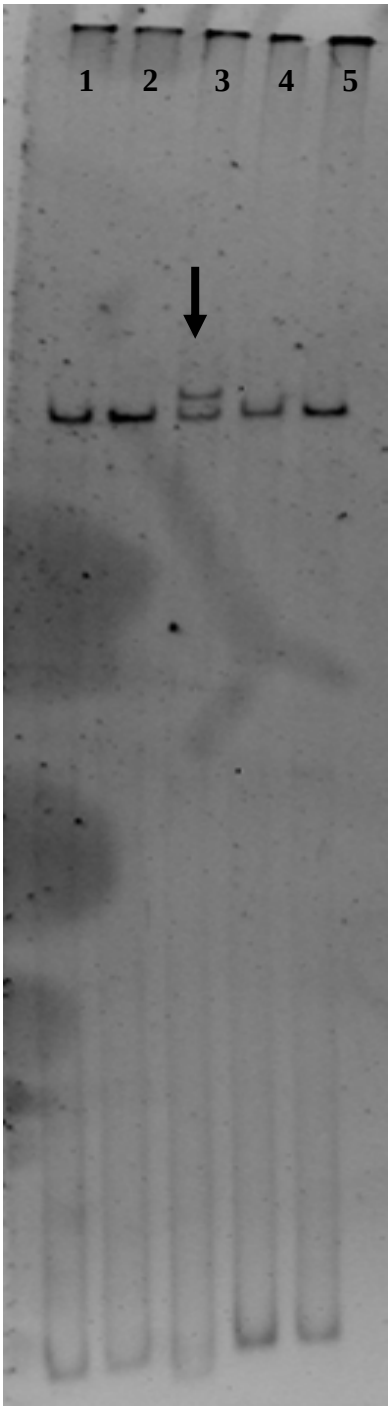

Figure 4.4 *BRCA1* exon 11K fragment. SSCP/HA polyacrylamide gel stained with EtBr. An individual with the S1140G variant has been marked (lane 3).

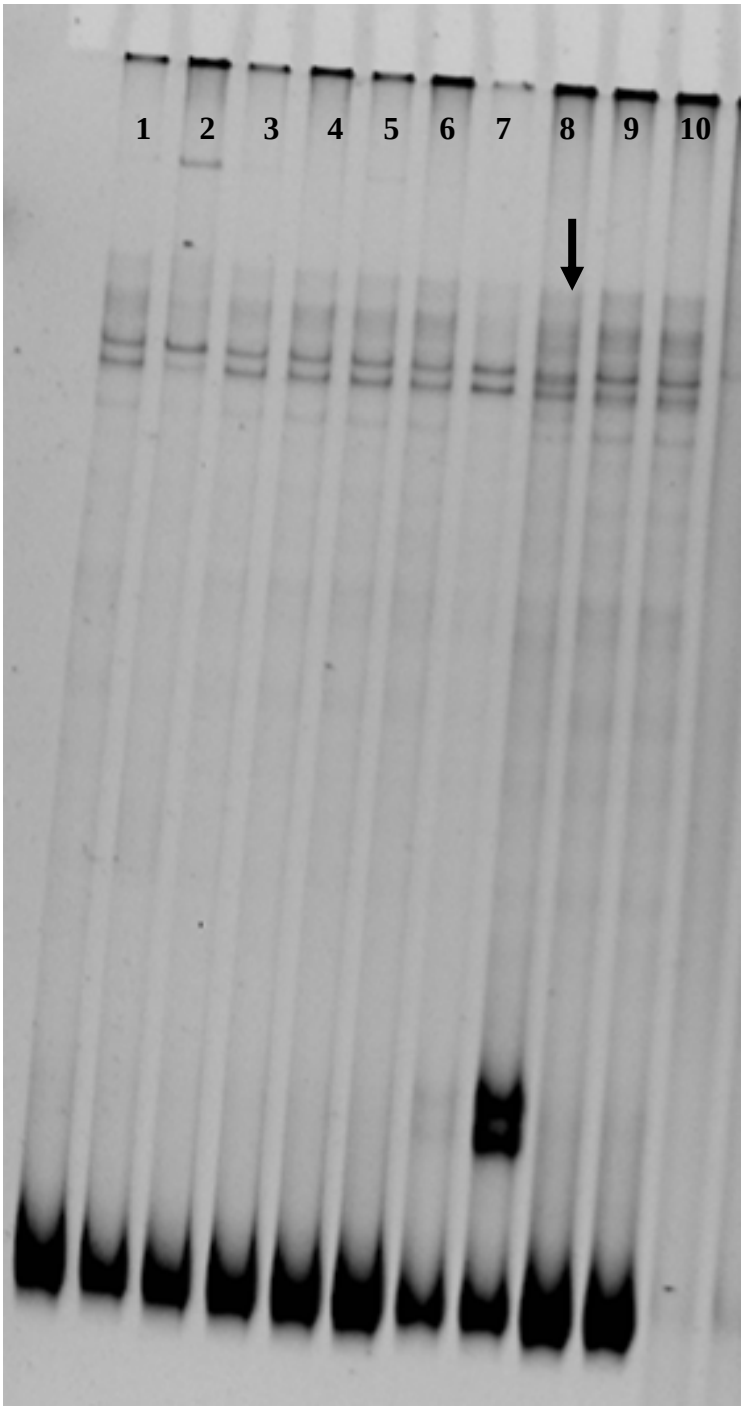

Figure 4.5 *BRCA1* exon 11C fragment. SSCP/HA polyacrylamide gel stained with EtBr. An individual with the 1623delTTAAA allele has been marked (lane 8).

4.1.3 Melt-point analysis by microplate-array diagonal-gel electrophoresis (Melt-MADGE)

Melt-MADGE analysis was done for exon 11 by INM Day. The fragments which have been reported to show variant migration were subsequently sequenced. Results have been included in Table 4.1. Three variants in *BRCA1* exon 11 (D693N, S694S and L771L) were detected with both Melt-MADGE analysis and SSCP/HA. The three other exon 11 variants detected with Melt-MADGE (T703T, N723D and T1349M) could not be detected with SSCP/HA. The nine remaining exon 11 variants were only detected with SSCP/HA.

4.1.4 DNA sequencing

Samples showing SSCP/HA variants were re-amplified and electrophoresed on an agarose gel before DNA sequencing was performed. This was done to ensure amplification and also to assist in calculating the concentration in order to obtain the best possible sequencing results.

The chromatographic tracings were visually inspected to exclude missed heterozygotes due to unequal amplification of the two alleles, after which multiple alignments were performed using the ClustalW program (version 1.4) in the BioEdit Sequence Alignment Editor software (Hall 1999). A total of 25 variants were detected (21 in this study) (Table 4.1). Four of the variants (5451+88T>C, 5526-76delT, 5585T>C, 5587-55C>G) could not be detected with SSCP/HA, but were identified after DNA sequencing of random samples to confirm amplification of the wild-type sequence. These variants were in return detected in all of the samples sequenced and it was established that these changes are either in the intronic regions or did not cause amino acid changes (indicated in Table 4.1). The high cost of direct sequencing of all the patients was therefore not justified. It is thus not possible to determine allele frequencies. Only one of these variants, 5451+88T>C, was also present in the homozygous form.

Most of the detected variants have been described in the BIC database (Breast Cancer Information Core http://research.nhgri.nih.gov/projects/bic), either as disease-causing mutations (M), benign polymorphisms (P) or unclassified variants (UV). Five of the variants detected in this study have not been described elsewhere and will be regarded as novel variants. Two of these, G677G and H1822H do not cause an amino acid change. The remaining three (5451+88T>C, 5526-76delT and 5587-55C>G) falls within intronic regions. The exon 11 variants identified in a previous study (Appendix C,

Table 4.1 Summary of *BRCA1* variants detected in the study population.

Detection Method	Exon/ Intron	Nucleotide number & base change	Amino acid change	Mutation Effect*	Number of Samples
SSCP/HA	8	622A>C	K168T**	Not in database	1
SSCP/HA	11	1623_1627delTTAAA	STOP503**	M	1
SSCP/HA	11	2150A>G	G677G	Not in database	1
Melt-MADGE & SSCP/HA	11	2196G>A	D693N	P	2
Melt-MADGE & SSCP/HA	11	2201C>T	S694S	P	4
Melt-MADGE	11	2228A>G	T703T	UV	1
Melt-MADGE	11	2286A>G	N723D	UV	1
Melt-MADGE & SSCP/HA	11	2430T>C	L771L	P	Refer to Table 4.3 for allele frequencies
SSCP/HA	11	2731C>T	P871L	P	Refer to Table 4.3 for allele frequencies
SSCP/HA	11	2760G>T	E881X**	Not in database	1
SSCP/HA	11	2933A>G	P938P	P	1
SSCP/HA	11	3232A>G	E1038G	P	3
SSCP/HA	11	3238G>A	S1040N	UV	1
SSCP/HA	11	3537A>G	S1140G	UV	1
SSCP/HA	11	3667A>G	K1183G	P	5
Melt-MADGE	11	4165C>T	T1349M	UV	1
SSCP/HA	18	5271+66G>A (IVS18+G>A)	Non-coding	P	4
SSCP/HA	18	5271+85delT (IVS18+85delT)	Non-coding	UV	1
SSCP/HA	20	5313delC	STOP1764**	Not in database	1
DS	21	5451+88T>C (IVS21+88T>C)	Non-coding	Not in database	All samples sequenced (n= 8)
SSCP/HA	22	5525+8T>C (IVS22+8T>C)	Non-coding	P	2
DS	22	5526-76delT (IVS23-76delT)	Non-coding	Not in database	All samples sequenced (n= 5)
DS	23	5585T>C	H1822H	Not in database	All samples sequenced (n= 5)
DS	23	5587-55C>G	Non-coding	Not in database	All samples sequenced (n= 5)
SSCP/HA	24	3'UTR36C>G	Non-coding	UV	5

P = polymorphism, UV = unclassified variant, M = disease causing mutation

*Classification of variants according to the BIC database (last accessed 2005/08).
**Variants identified in previous study (Appendix C, Scholtz *et al.* 2003).

Scholtz *et al.* 2003) were independently confirmed during this study. The sequencing chromatogram for mutation 1623delTTAAA is shown in Figure 4.6. All variants were verified by bi-directional sequencing.

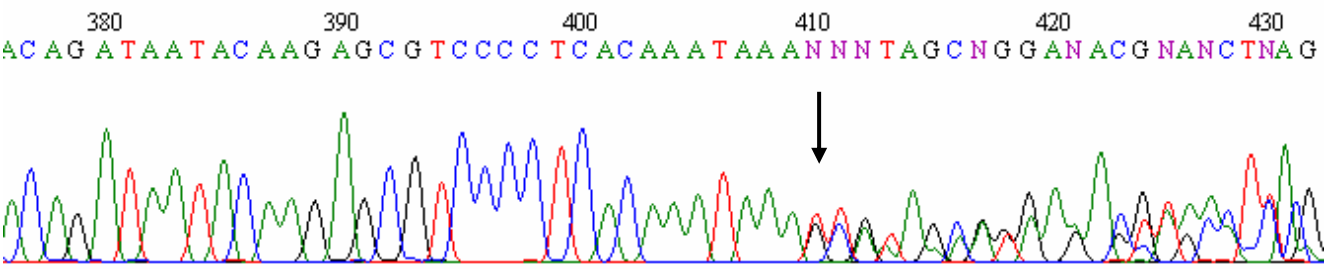

Figure 4.6 Results obtained from the automated sequencing in the sense (5'→3') direction of the *BRCA1* exon 11C fragment of a heterozygous individual containing the 1623delTTAAA mutation.

The human BRCA1 protein shows 56% sequence identity to the mouse (*Mus musculus*) Brca1 protein and 74% identity to the dog (*Canis familiaris*) Brca1 protein. Amino acid conservation in these mammals were established at selected positions. This was done for the unclassified variants which will result in an amino acid change. The various residues at the selected positions are listed in Table 4.2. The only residue conserved between all three species, is asparagine, which is changed by the N723D variant to aspartic acid.

Table 4.2 Amino acids identified in the wild-type sequence of the BRCA1 protein for three mammalian species. The amino acids in question are indicated and forty flanking residues are included. Amino acids conserved across all species have been shaded.

Variant	Organism	Amino acid sequence
K168T*	Human	QETSLSVQLSNLGTVRTLRTKQRIQPQKTSVYIELGSDSSE
	Mouse	-KDSLGVQLSNLGIVRSVKKNRQTQPRKKSVYIELDSDSSE
	Dog	-ETSLSVQLSNLGIVRSLRTKQQIQPQNKSVYIELGSDSSE
N723D	Human	TNAPGSFTKCSNTSELKEFVNPSLPREEKEEKLETVKVSNN
	Mouse	MNKAGLLTSCSSPRKSQGPVNPSPQRTG-TEQLETRQMSDS
	Dog	TNVSGFFANYSSSSKPQECINPGLRREEIEESRRMTQVSDS
S1040N	Human	PSTVSTISRNNIRENVFKEASSSNINEVGSSTNEVGSSINE
	Mouse	QSTVHTVSLNN-RGNACQEAGS--------------GSIHE
	Dog	QSTVSTISQNNIRECASKEVGSSSVNEVVSSTNEVGSSVNE
T1349M	Human	VNTDFSPYLISDNLEQPMGSSHASQVCSETPDDLLDDGEIK
	Mouse	VCADFSPCLFSDHLEQSMSG-KVFQVCSETPDDLLDDVEIQ
	Dog	VSADFSPCLISDNPEQPMGSSRSSQVCSETPDDLLNGDKIK

*Variant identified in previous study (Appendix C, Scholtz *et al*. 2003).

4.1.5 Statistical analysis

The majority of the reported variants was only detected in five samples or less and was therefore regarded as rare. However, two variants, L771L and P871L were observed at a high frequency. Genotype frequencies for the L771L and the P871L variants have been calculated and are shown in Table 4.3.

Table 4.3 Genotype frequencies for the L771L and P871L variants in the study population.

Polymorphism	Genotype	Population		
		Caucasian n=19 (%)	Mixed Ancestry n=29 (%)	Total n=48 (%)
L771L	TT	7 (36.8%)	16 (55.2%)	23 (47.9%)
	TC	9 (47.4%)	12 (41.4%)	21 (43.8%)
	CC	3 (15.8%)	1 (3.4%)	4 (8.3%)
P871L	CC	7 (36.8%)	0 (0.0%)	7 (14.6%)
	CT	9 (47.4%)	13 (44.8%)	22 (45.8%)
	TT	3 (15.8%)	16 (55.2%)	19 (39.6%)

Fifty random controls of the Mixed Ancestry population were genotyped for the novel variant K168T (identified in a previous study by Scholtz *et al.* 2003 (Appendix C)). Genomic DNA was amplified with *BRCA1* exon 8 primers (forward 5'-AGCTGACTGATG-3' and reverse 5'-AAATTCACTTCC-3'), PCR Program A (refer to section 3.2.4) at an annealing temperature of 55°C. The resulting amplicons of 338bp were analysed together with the positive control for the variant with SSCP/HA (refer to section 3.2.5). None of the control individuals are carriers of the threonine allele.

4.2 THE *BRCA2* GENE

The study population was screened for the *BRCA2* Ashkenazi Jewish founder mutation 6174delT before this study commenced by CL Scholtz and MJ Kotze (unpublished data). The mutation was not detected.

4.2.1 Polymerase chain reaction (PCR) amplification

The coding exons of the *BRCA2* gene were amplified in 39 fragments, including the five overlapping fragments for exon 10 and the seven overlapping fragments for exon 11. A nested PCR was performed for exons 15, 20 and 22, as amplification of the individual exons yielded either non-specific products or no amplification at all. Adjacent exons were amplified together with the exon in question in an initial reaction. The product was used as template for a second reaction (refer to Table 3.5).

As for *BRCA1*, all amplicons were resolved on an agarose gel to verify amplification of the correct fragment before further analysis either by SSCP/HA or direct DNA sequencing. Figure 4.7 and Figure 4.8 show examples of such gels, with exon 10 and exon 11 fragments respectively. As can be seen in these figures, fragment sizes were ~350bp for exon 10 fragments and ~850p for exon 11 fragments. All PCR reactions were successfully optimized. No primer dimers were visible on the agarose gels.

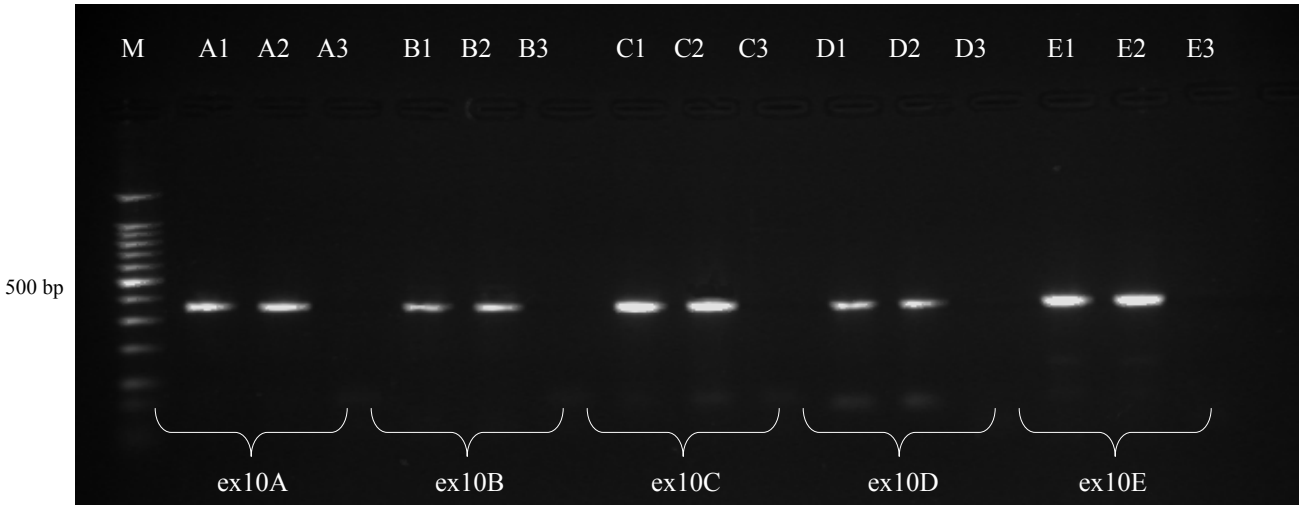

Figure 4.7 *BRCA2* exon 10 fragments. A 1.5% agarose gel, resolved for 1 hour at 100V in 1XTBE buffer and stained with 0.5µg/µl EtBr. The photograph was taken after exposure to ultra- violet light. A 100bp molecular marker is loaded in the first lane (M), with the 500bp band indicated. Lanes A1 to E2 represent the different fragments for exon 10, with the negative control loaded in the third lane of each fragment (A3 to E3).

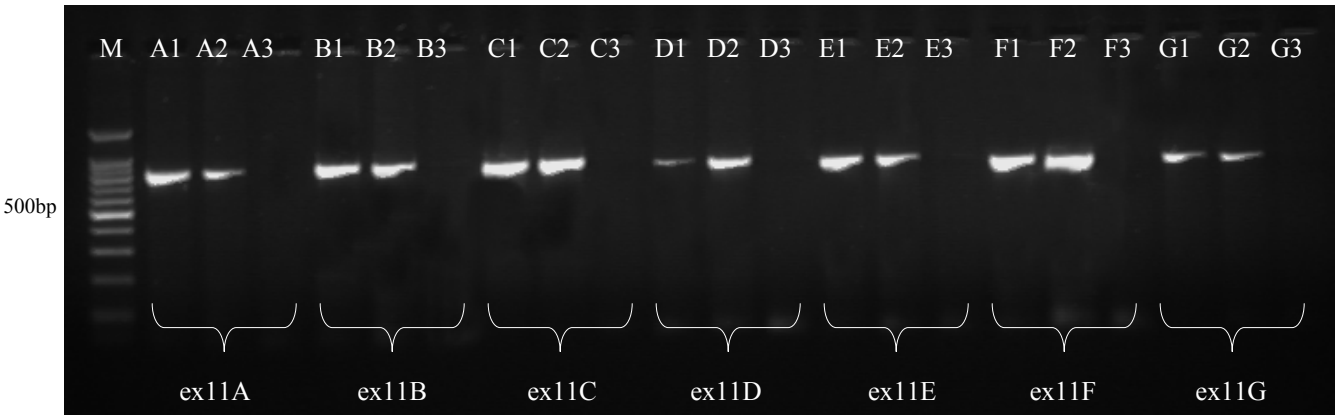

Figure 4.8 *BRCA2* exon 11 fragments. A 1.5% agarose gel, resolved for 1 hour at 100V in 1XTBE buffer and stained with 0.5µg/µl EtBr. The photograph was taken after exposure to ultra violet light. A 100bp molecular marker is loaded in the first lane (M), with the 500bp band indicated. Lanes A1 to G2 represent the different fragments for exon 11, with the negative control loaded in the third lane of each fragment (A2 to G3).

A few of the *BRCA2* exon 11 fragments were not analysed for some of the patients. This was due to poor quality of the PCR amplification products after repeated attempts were made of obtaining suitable template for DNA sequencing. Due to insufficient amounts of DNA samples for these individuals, any further efforts had to be abandoned. This included one patient for each of exons 11A, 11C and 11D for whom no results are available, as well as ten patients for exon 11E which was only sequenced in the sense direction, still allowing for sequence analysis.

4.2.2 Single strand conformation polymorphism / heteroduplex analysis (SSCP/HA)

As for *BRCA1*, only one SSCP gel electrophoresis system was used to screen the *BRCA2* gene for sequence variations. All fragments except exon 9 were resolved on a 12% (w/v) polyacrylamide gel supplemented with 7.5% (w/v) urea at 4°C. Exon 9 fragments showed improved resolution on a 20% (w/v) polyacrylamide gel without glycerol or urea, electrophoresed at room temperature. Representative gels are shown in Figures 4.9, 4.10 and 4.11. The banding pattern for the N372H variant is shown in Figure 4.11. Band intensities were not considered as PCR products were not diluted to have similar concentrations before being subjected to electrophoresis. Band intensities on the printed polyacrylamide gel images may therefore vary, e.g. lanes 5 and 11 in Fig 4.10. Analyses were performed on enlarged images on a standard computer monitor in order to enable compensation for differences in band intensities.

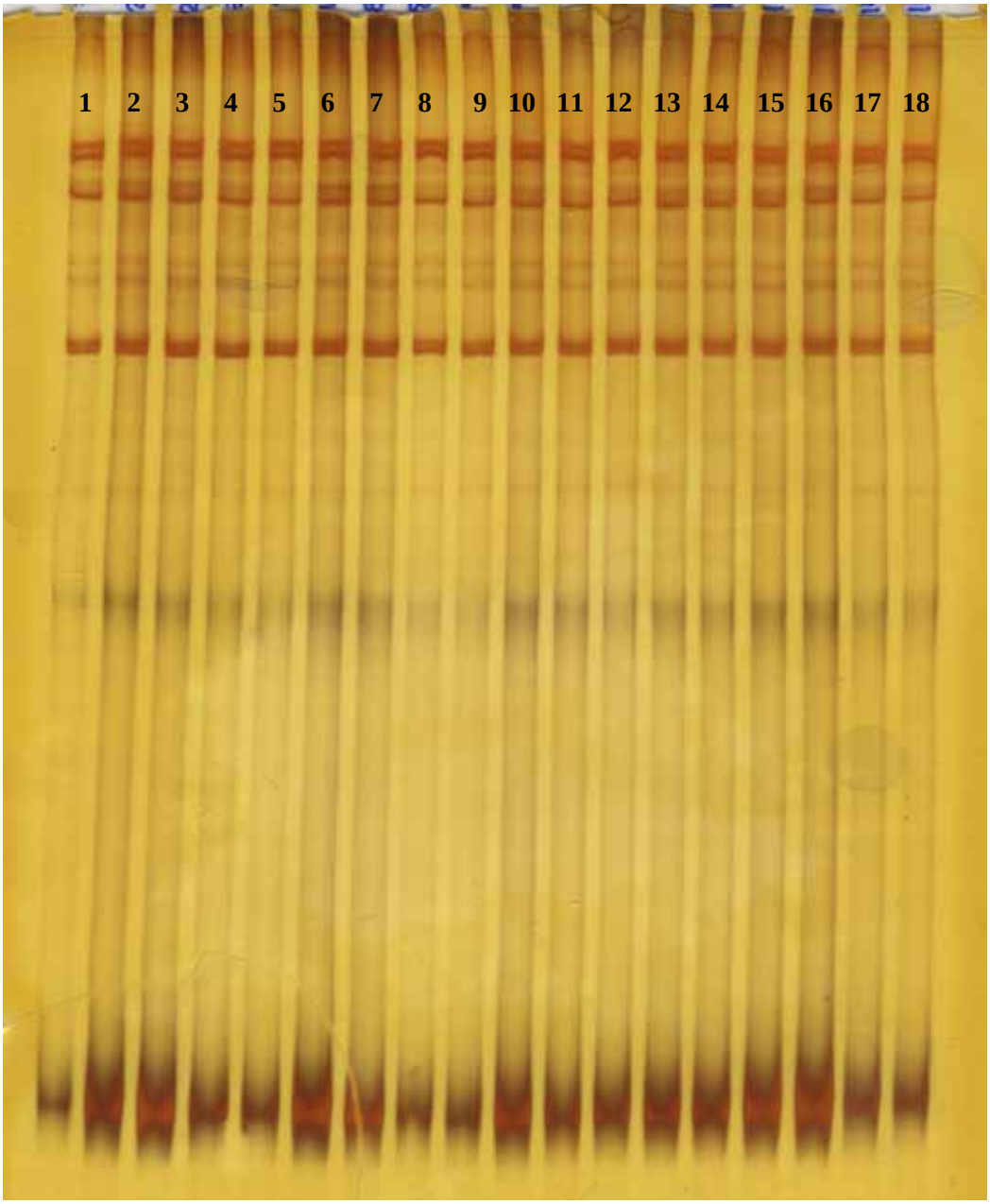

Figure 4.9 *BRCA2* exon 16. SSCP/HA gel polyacrylamide gel stained with $AgNO_3$. No variants were detected in this fragment for any of the patients.

Figure 4.10 *BRCA2* exon 12. SSCP/HA polyacrylamide gel stained with $AgNO_3$. No variants were detected in this fragment for any of the patients.

Figure 4.11 *BRCA2* exon 10B. SSCP/HA polyacrylamide gel stained with EtBr. Individuals heterozygous for the N372H variant can be seen in lanes 2, 3, 6 and 7. All other lanes contain wild-type sequences.

4.2.3 DNA sequencing

Samples showing SSCP/HA variants were re-amplified and electrophoresed on an agarose gel before direct DNA sequencing. Exon 11 fragments were directly sequenced. The chromatographic tracing were visually inspected before multiple alignments were performed using the ClustalW program (version 1.4) in the BioEdit Sequence Alignment Editor (Hall 1999). A total of 29 variants were detected (Table 4.4).

The BIC database was searched for the detected variants and classified according to reports submitted to this database. Most of the detected variants have been described in the BIC database, either as disease-causing mutations, benign polymorphisms or unclassified variants. Nine of the detected variants have not been described elsewhere and will be referred to as novel variants. Four of these are non-coding variants (5'UTR199T>G, 5'UTR212G>A, 549-26C>T, 7236-27A>T). Two variants (L775L, D3341D) do not cause an amino acid change. One of the novel variants does cause an amino acid change (N2447D) and one variant is a 2 base pair insertion (6677insTA) (see Figure 4.12 for the sequencing results). The ninth unreported variant, N1600del, is an inframe three base pair deletion, removing asparagine from the peptide. One previously reported mutation has been identified in three patients, 8162delG (Figure 4.14). All the variants that were encountered will be discussed in detail in Chapter 5. The chromatogram for two alleles of the N372H variant is shown in Figure 4.13. Since this variant can be detected with SSCP/HA, only a few selected samples have been sequenced to serve as controls for determining the genotype of the remaining samples.

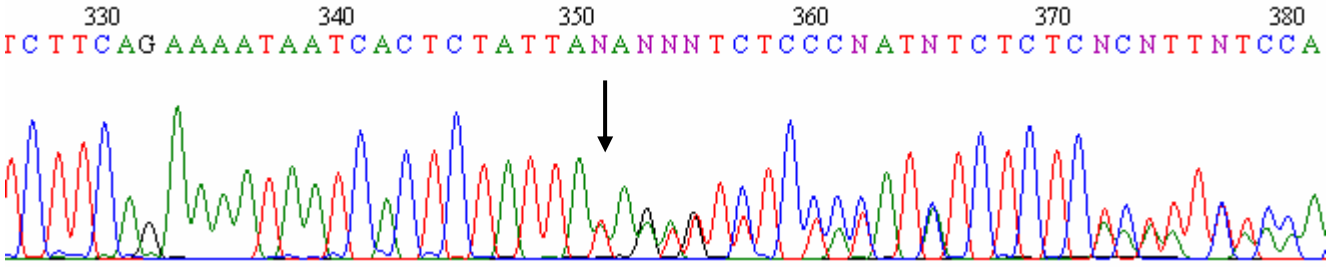

Figure 4.12 Results obtained from the automated sequencing in the sense (5'→3') direction of the *BRCA2* exon 11G fragment of a heterozygous individual containing the 6677insTA mutation.

Table 4.4 Summary of *BRCA2* variants detected in the study population.

Detection Method	Exon/ Intron	Nucleotide number & base change	Amino acid change	Mutation Effect*	Number of Samples
SSCP/HA	2	5'UTR199T>G	Non-coding	Not in database	1
SSCP/HA	2	5'UTR212G>A	Non-coding	Not in database	3
SSCP/HA	3	549-26C>T (IVS4-26C>T)	Non-coding	Not in database	2
SSCP/HA	10	1093A>C	N289H	P	2
SSCP/HA	10	1342C>A	N372H	P	Refer to Table 4.4 for allele frequencies
DS	11	2457T>C	H743H	P	1
DS	11	2511T>A	L775L	Not in database	1
DS	11	3111G>A	Q961Q	P	1
DS	11	3199A>G	N991D	P	1
DS	11	3624A>G	K1132K	P	Refer to Table 4.4 for allele frequencies
DS	11	3673A>G	M1149V	UV	1
DS	11	4035T>C	V1269V	P	Refer to Table 4.4 for allele frequencies
DS	11	4086_4088delAAA	K1285del	UV	1
DS	11	4296G>A	L1356L	P	1
DS	11	4791G>A	L1521L	P	2
DS	11	5026_5028delAAT	N1600del	Not in database	1
DS	11	5642A>G	N1805S	P	2
DS	11	5972C>T	T1915M	P	1
DS	11	6640G>T	V2138F	P	2
DS	11	6677_6678insTA or 6675_6676dupTA	STOP2167	Not in database	2
DS	11	6741C>G	V2171V	P	2
SSCP/HA	13	7236-27A>T (IVS14-27A>T)	Non-coding	Not in database	3
SSCP/HA	14	7470A>G	S2414S	P	3
SSCP/HA	14	7567A>G	N2447D	Not in database	1
SSCP/HA	16	8035-15T>C (IVS17-15T>C)	Non-coding	P	2
SSCP/HA	17	8162delG**	Stop2647	M	3
SSCP/HA	25	9486-17T>C (IVS26-17T>C)	Non-coding	P	1
SSCP/HA	27	10251C>T	D3341D	Not in database	5
SSCP/HA	27	10462A>G	I3412V	UV	1

P = polymorphism, UV = unclassified variant, M = disease causing mutation

*Classification of variants according to the BIC database (last accessed 2005/07).
**Mutation detected in the two male patients from the study group.

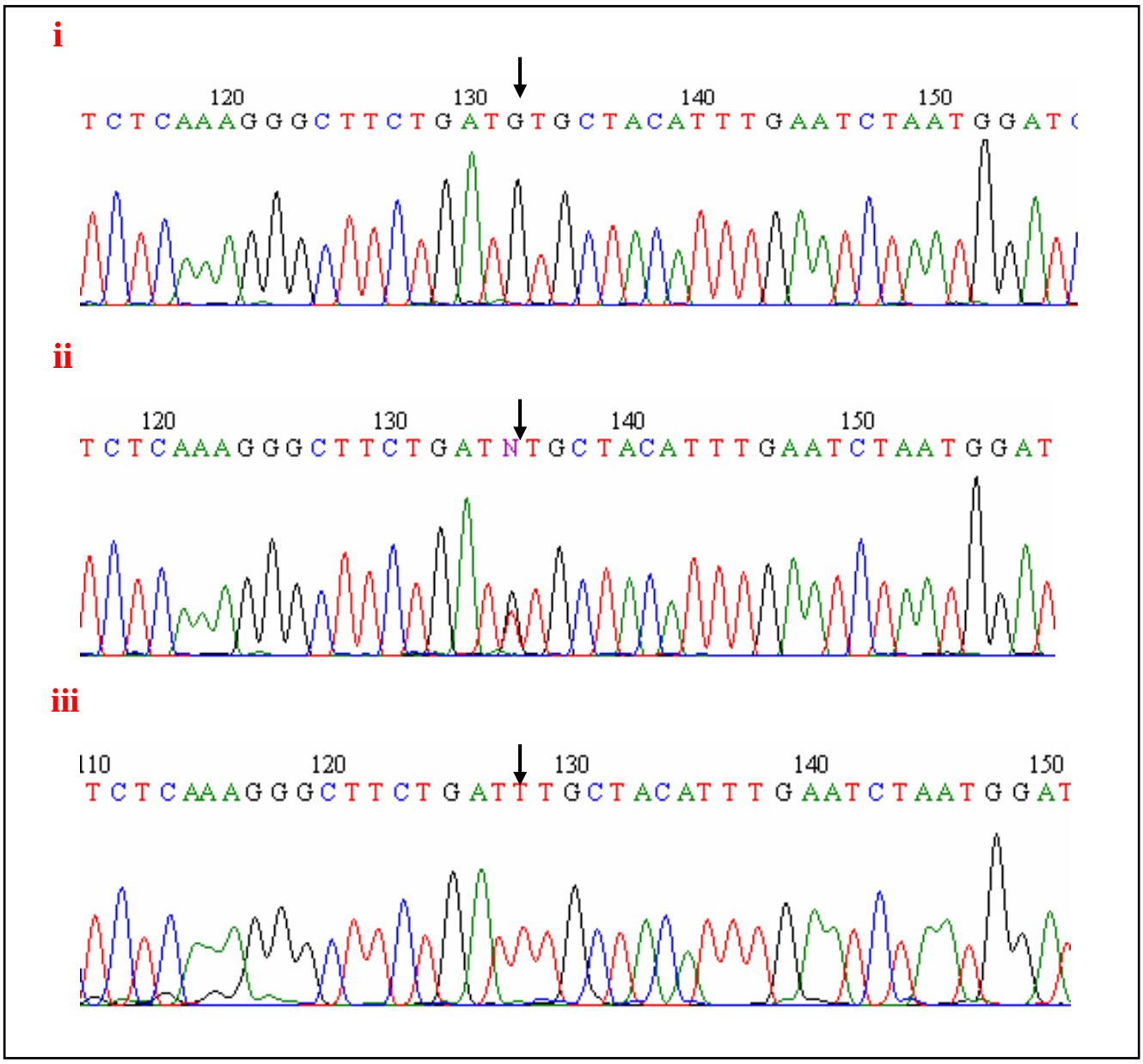

Figure 4.13 Results obtained from the automated sequencing in the antisense (3'→5') direction of the *BRCA2* exon 10B fragment showing the N372H variant for i) individual homozygous for the G allele (His), ii) heterozygous individual and iii) individual homozygous for the T allele (Asn).

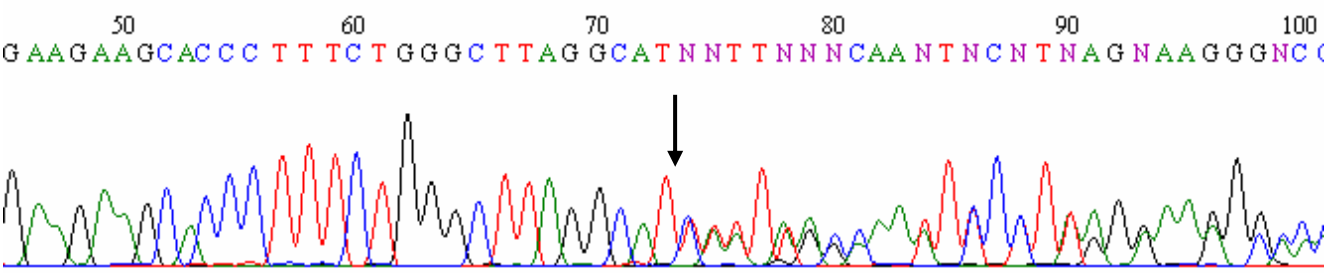

Figure 4.14 Results obtained from the automated sequencing in the antisense (3'→5') direction of the *BRCA2* exon 17 fragment of a heterozygous individual containing the 8162delG mutation.

The human BRCA2 protein shows 57% sequence identity to the mouse (*Mus musculus*) Brca1 protein and 69% identity to the dog (*Canis familiaris*) Brca1 protein. Amino acid conservation these mammals were established at selected positions. This was done for the unclassified variants which will result in an amino acid change or an inframe deletion or insertion. The various residues at the selected positions are listed in Table 4.5. The amino acid is only conserved between all three species for one of the selected variants, K1285del, a deletion of lysine.

Table 4.5 Amino acids identified in the wild-type sequence of the BRCA2 protein for three mammalian species. The amino acids in question are indicated and forty flanking residues are included. Amino acids conserved across all species have been shaded.

Variant	Organism	Amino acid sequence
M1149V	Human	QFRKPSYILQKSTFEVPENQMTILKTTSEECRDADLHVIMN
	Mouse	QFRKPSHIAQN-TSEVPGNQMVVLSTASKEWKDTDLHLPVD
	Dog	QFRKPSHIIQKNPFEMPENQLTILNSTSKEWKDDDLHLTTN
K1285del	Human	DSVVSMFKIENHN-DKTVSEKNNKCQLILQNNIEMTTGTFV
	Mouse	DSVASVFKIKKQNTEKSFDEKSSKCQVTLQNNIEMTTCIFV
	Dog	DS-VSMIQIEDCN-DKNLNEKNNKCRLILQNNIEMTTDIFV
N1600del	Human	CETIEITAAPKCKEMQNSLNNDKNLVSIETVVPPKLLSDNL
	Mouse	YEKIEVTAS-KCEEMQNFVSKETEMLPQQ--------NYHM
	Dog	CGTTEITTTPEYEETHSSLEKKKLVSNEIAALRPRLLSDNL
N2447D	Human	LEENRQK-QNIDGHGSDDSKNKINDNEIHQFNKNNSNQAAA
	Mouse	LEGKNQK-------STDGDREDGNDSHVRQFNK--------
	Dog	LEKNKQNSKDIDELGSGDSEKNINDSGIHQLKKNNSNQAAT
I3412V	Human	KEQESSQASTEECEKNKQDTITTKKYI----------
	Mouse	VGPRSRKESLRDCRGDSSEKLAVES----------
	Dog	RGPESPQACTRKREPRVQNTSDLKRTSLRLQRQQTQK

4.2.4 Statistical analysis

Three variants were observed at an elevated frequency in the sample population. The genotype frequencies for the N372H, K1132K and V1269V variants have been calculated and are shown in Table 4.6. Hardy-Weinberg equilibrium (HWE) for one variant, N372H, was tested for using Fisher's exact test. The computer software Tools for Population Genetic Analysis, TFPGA (Miller and Mark 1997) was used to calculate the test statistic and *P* values. Both the Caucasian (*P*=0.3177) and the Mixed Ancestry population (*P*=0.3899) demonstrated Hardy-Weinberg equilibrium at a 95% significance level. When calculating probability values for the total study population, including and excluding the male breast cancer patients, Hardy-Weinberg equilibrium was still demonstrated (*P*=0.1013 and *P*=0.1723 respectively).

Table 4.6 Genotype frequencies for the N372H, K1132K and the V1269V variants in the study population.

Polymorphism	**Genotype**	**Population**		
		Caucasian n=19 (%)	**Mixed Ancestry n=29 (%)**	**Total n=48 (%)**
N372H	AA	6 (31.6%)	14 (48.3%)	20 (41.7%)
	AC	12 (63.2%)	14 (48.3%)	26 (54.2%)
	CC	1 (5.3%)	1 (3.4%)	2 (4.2%)
K1132K	AA	6(31.6%)	21(72.4%)	27(56.3%)
	AG	13(68.4%)	8(27.6%)	21(43.8%)
	GG	0(0.0%)	0(0.0%)	0(0.0%)
V1269V	TT	13(68.6%)	22(44.8%)	35(72.9%)
	TC	6(31.6%)	6(20.7%)	12(25.0%)
	CC	0(0.0%)	1(3.4%)	1(2.1%)

Fifty random controls of the Mixed Ancestry population were genotyped for the novel variant K1600del. Genomic DNA was amplified with *BRCA2* exon 11E primers (refer to section 3.2.4) where after the resulting amplicons of 875bp were analysed together with the positive control for the variant

with SSCP/HA (refer to section 3.2.5). Despite the size of the fragment, the three base pair deletion could be distinguished from the wild-type allele. The deletion was not detected in any of the control samples.

4.3 THE *CHEK2**1100delC VARIANT

4.3.1 Polymerase chain reaction (PCR) amplification

Exon 10 of the *CHEK2* gene was amplified, yielding a 245bp fragment. Figure 4.15 shows an agarose gel photo of amplification products for some of the samples. A 100bp DNA molecular marker was always included as well as a negative control (i.e. without template DNA). Primer dimers were not a concern as it does not influence the results when using SSCP/HA as a screening method. As discussed in previous chapters, the *CHEK2* gene has various homologous sequences spread across the genome. One of these pseudogenes, located on chromosome 15, is the exact same length as exon 10. It was therefore not possible to distinguish between the two fragments on an agarose gel, but only after electrophoresis of the SSCA/HA polyacrylamide gel. Figure 4.16 shows the various products obtained with different PCR annealing temperatures. The PCR was optimized according to these results. Amplification was optimal using Program C, with 10 cycles at 65°C annealing and 30 cycles at 60°C annealing. At these annealing temperatures, difficulties were experienced when attempting to amplify some of the samples, as is apparent from lane four in Figure 4.16. This was resolved by increasing the gDNA concentrations.

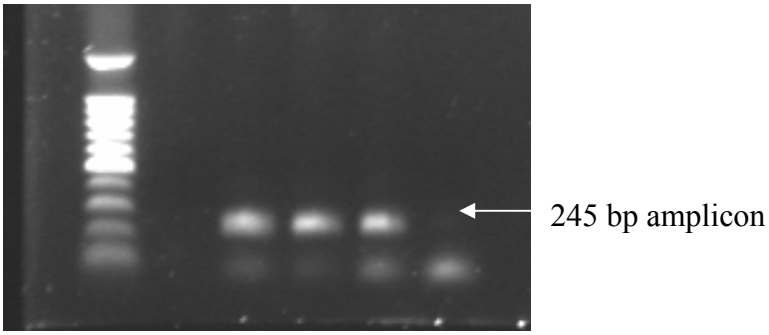

Figure 4.15 A 1% agarose gel, resolved for 1 hour at 100V in 1XTBE buffer and stained with 0.5µg/µl EtBr. The photograph was taken after exposure to ultra violet light. A 100bp molecular marker is loaded in the first lane (M). Lanes 1 to 3 represent the exon 10 fragment of the *CHEK2* gene, with the negative control loaded in lane 4.

4.3.2 Single strand conformation polymorphism / heteroduplex analysis (SSCP/HA)

Two different SSCP/HA gel systems were used to test the *CHEK2* fragment for the presence of the 1100delC mutation. The first system tested was the Mighty Small gel (10% and 15% (w/v) polyacrylamide gel). The other system tested had different gel dimensions and composition (12% (w/v) polyacrylamide gel containing 7.5% (w/v) urea). The second system allowed for electrophoresis to be performed for a longer period of time, improving separation of the bands. Neither of the gel systems used showed any variation in the banding pattern. A positive control sample was not available. As a deletion would be expected to cause a conformational change in the DNA which will alter the motility of the fragment, it can be concluded that the 1100delC allele (nor any other exon 10 variation) is not present in the breast cancer population sampled.

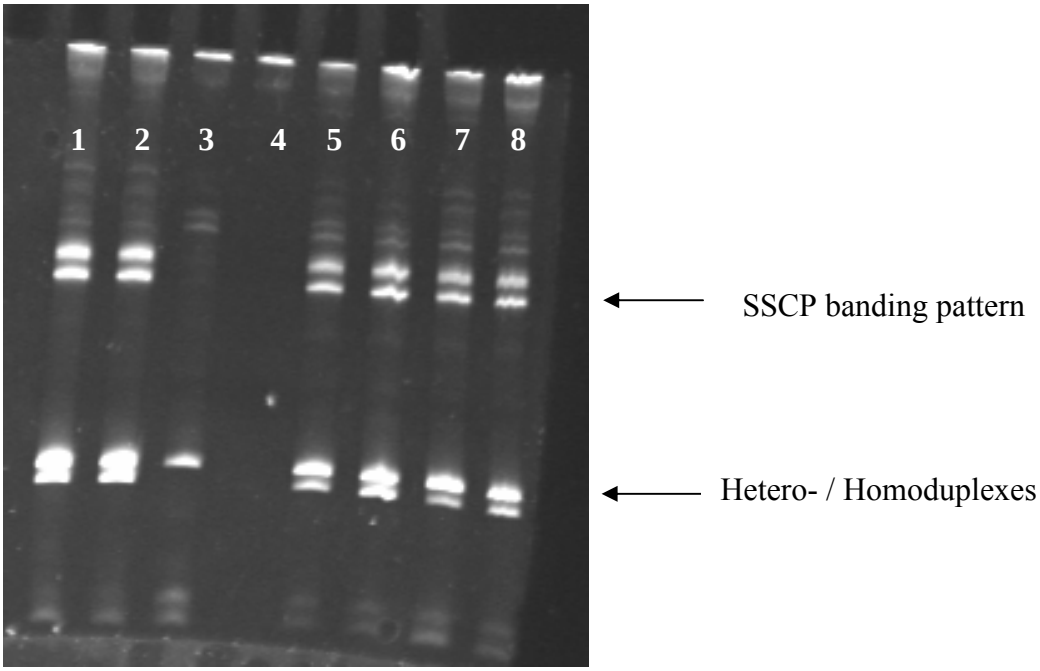

Figure 4.16 Electrophoresis performed on a Mighty Small polyacrylamide gel to determine which program was optimal for PCR amplification. Lane 1 and 2: touch-down PCR program D (65→60°C); lane 3 and 4: two-step PCR program C (65/60°C); lane 5 and 6: touch-down PCR program D (64→59°C); lane 7 and 8: two-step PCR program C (64/60°). Lane 3 is indicative of a single amplification product, all other lanes represent non-specific amplification of homologous sequences. Amplification was unsuccessful of the sample loaded in lane 4.

4.3.3 DNA sequencing

Five samples were subjected to direct DNA sequencing to confirm amplification of the *CHEK2* gene and not any of the homologous sequences. The discrepancies between the gene and the pseudogene are marked in Figure 4.17. Sequencing results clearly show amplification of the correct fragment. The DNA sequencing data supported the SSCP/HA results, which do not suggest the presence of the *CHEK2**1100delC mutation in the study population.

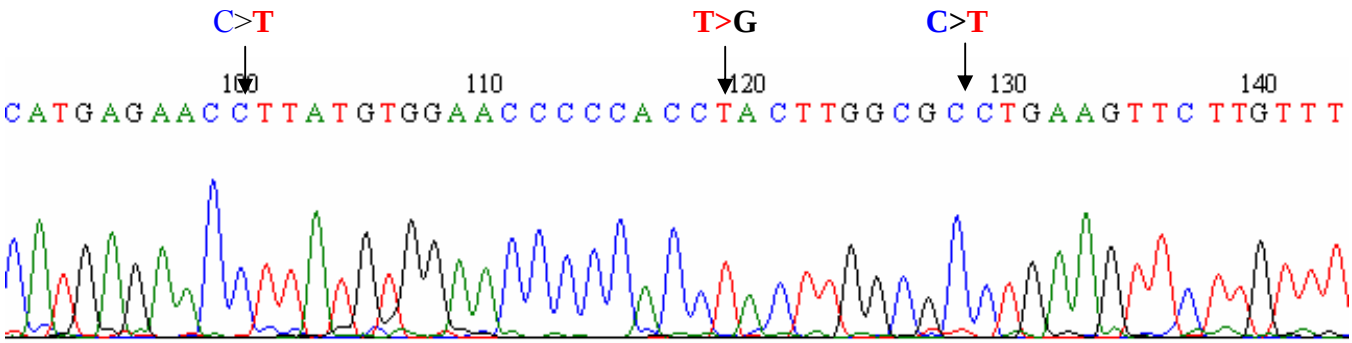

Figure 4.17 Results obtained from the automated sequencing in the sense (5'→3') direction of the *CHEK2* exon 10 fragment with. The bases discriminating the exon 10 fragment on chromosome 22 from the pseudogene on chromosome 15 are indicated.

4.4 Summary of results

In this study, 5 899bp (4139bp coding and 1760bp non-coding) of gDNA have been screened for the *BRCA1* gene in 48 individuals. SSCP/HA was performed for the coding and adjacent non-coding regions (intron-exon boundaries) in 22 fragments. A total of 22 variants have been detected (plus three variants from the previous study by C Scholtz and MJ Kotze). Fifteen of these variants have been detected in coding regions. The ratio of coding to non-coding areas screened is 2.35**:**1, and the ratio of variants detected in the coding to non-coding areas is 2.14**:**1. Coding areas therefore appear to be as polymorphic as non-coding areas.

For the *BRCA2* gene, 14 799bp (10257bp coding and 4542bp non-coding) of gDNA have been screened in 48 individuals. SSCP/HA was performed in 32 fragments of coding regions and splice sites. Direct DNA sequencing was done for 7 fragments (exon 11). A total of 29 variants have been detected. Twenty-three of these were in the coding regions. The ratio of coding to non-coding areas screened is 2.26**:**1, and the ratio of variants detected in the coding areas in relation to variants detected in the non-coding areas is 3.83**:**1. This suggests that the coding areas are more polymorphic than the non-coding areas. It should, however, be considered that the non-coding areas were predominantly screened with SSCP/HA, which is less sensitive than DNA sequencing, the method employed to screen exon 11.

Since only exon 10 of the *CHEK2* gene was screened and the *CHEK2**1100delC mutation was not detected in the study population, no deductions can be made as to the role this or any other *CHEK2* variant has in the pathogenesis of breast cancer in South African women and men.

5. DISCUSSION

The primary objectives of this study were to determine the *BRCA1* and *BRCA2* mutation spectrum in South African breast cancer probands and to ascertain whether South African breast cancer patients are carriers of the *CHEK2**1100delC mutation. Forty-eight South African breast cancer patients were screened for *BRCA1* and *BRCA2* mutations using SSCP/HA analysis and DNA sequencing. Deleterious *BRCA1* mutations were identified in three patients (6.3%) and *BRCA2* mutations in five patients (10.4%), together accounting for 16.7% of breast cancer in the study population. In addition, a number of benign polymorphisms and variants of unknown functional significance were identified. Our findings do not suggest the presence of the *CHEK2* mutation in South African breast cancer patients.

Variants have been divided into different categories, firstly depending on whether it has been reported in the BIC database (Breast Cancer Information Core http://research.nhgri.nih.gov/projects/bic), thereafter as non-coding (nucleotide changes within intronic and in 5'- or 3'-untranslated regions), benign polymorphisms, unclassified variants or as pathogenic mutations. Although located in noncoding regions, critical sequences in the untranslated regions do influence stability of mRNA and therefore phenotype (Sachs 1993). Synonymous polymorphisms in both coding and non-coding regions can still affect gene function by altering the stability and splicing of the mRNA. Duan *et al.* (2003) demonstrated that certain combinations of synonymous mutations in the human dopamine receptor (*DRD2*) gene have functional consequences, namely decreasing mRNA stability and translation.

Missense mutations are generally assessed for possible pathogenicity according to the following criteria (Greenman *et al*. 1998): (**1**) segregation with the disease; (**2**) absence in ethnically matched controls; (**3**) nonconservative amino acid substitutions; (**4**) residues conserved in mammalian homologues of the *BRCA1* and *BRCA2* genes (e.g. murine and canine); or (**5**) occurrence within a conserved and possibly functional motif. Since no functional, case-control or family studies have been done for this study, pathogenicity of unclassified variants can only be inferred theoretically by means of (3), (4) and (5). Functional *in vitro* studies, utilizing mammalian cell line, can also be done to determine expression levels and to determine if the protein retains its various functions. For most variants which have been

reported in the BIC database, various entries exist. If in at least one of these entries a variant is classified as a benign polymorphism, it will be treated as such and it will be assumed that these claims can be substantiated by the respective research groups who submitted their data to the database.

The BIC database does not cross-reference to published studies. A literature search was therefore done in the NCBI PubMed database (www.ncbi.nlm.nih.gov/) for variants which have been reported. Available references for the first reports in the literature of these variants were included in the discussion which follows.

5.1 VARIANTS IDENTIFIED IN THE *BRCA1* GENE

5.1.1 Non-coding variants

Seven non-coding variants were identified in the *BRCA1* gene, including five single nucleotide changes and two one base pair deletions (Table 4.1). Two of these were reported as polymorphisms, 5271+85delT and 5525+8T>C (Futreal *et al.* 1994), and two were reported as unclassified variants, 5271+66G>A and 3'UTR36C>G (Newman *et al.* 1998). The 5271+66G>A variant was previously shown to be a frequent polymorphism (Futreal *et al.* 1994; Durocher *et al.* 1996; Malone *et al.* 1998) and was detected in four patients in our study population (8.3%). This polymorphism was initially designated PM5 by Futreal *et al.* (1994). The 5525+8T>C variant was identified in two of our patients (4.2%) and it was reported to be a benign polymorphism (Arena *et al.* 1996). A functional role for the unclassified variants has not been excluded. Three variants, 5451+88T>C, 5526-76delT and 5587-55C>G, have not been reported before. None of the non-coding variants are suspected to be involved in splicing as they are deep within the adjacent intronic regions. It should also be noted that the novel variants were detected in all the samples that were sequenced (five to eight samples in each instance), which is consistent with the theory of a frequent polymorphism rather than that of a rare mutation in the study population.

5.1.2 Coding variants

5.1.2.1 Benign polymorphisms

Seven single base pair substitutions were detected in the coding regions which are classified in the BIC database, classified as benign polymorphisms. Three of these do not cause an amino acid change, S694S, L771L (Friedman *et al.* 1994) and P938P (Newman *et al.* 1998), and are therefore not expected to effect the functions of the protein. The following polymorphisms do change the amino acid sequence, but have been shown by other research groups not to be functional, as they were also detected in control chromosomes: D693N (Durocher *et al.* 1996), P871L, K1183G (Miki *et al.* 1994) and E1038G (Friedman *et al.* 1994). P871L, K1183 (originally designated PM6 and PM7 by Miki *et al.* 1994) and E1038G have been shown to be frequent polymorphisms. The E1038G polymorphism was identified in three patients (6.3%) and the K1183G polymorphism in five patients (10.4%) from our study group. These variants have been detected at a much higher frequency by another research group in Canadian and American breast cancer patients (Durocher *et al.* 1996). They reported the E1038G polymorphism in 34% and the K1183G polymorphism in 31% of their patients. The proline allele of the P871L variant was present at a frequency of 0.37 in our Caucasian study population, which is in agreement with Durocher's study. They calculated the allele frequency in various population groups to be between 0.21 and 0.42. We did not detect the proline allele in the Mixed Ancestry population.

The following variants are under the *BRCA1* top 20 missense or synonymous mutations in the BIC database: S694S, L771L, P938P, K1183G and E1038G.

5.1.2.2 Pathogenic mutations

Three disease causing mutations have previously been identified in this study population. These results have been presented at a national congress (Appendix C, Scholtz *et al.* 2003). One of these mutations, 1623_1627delTTAAA, creates a stop codon at position 503, shortening the protein to only 26% of its full length (1863 amino acids). This mutation has been reported in the BIC database. The deletion has been identified in a female patient from Mixed Ancestry, diagnosed at 53 years of age. She reported one first degree relative with breast cancer. Another mutation, E881X, which has been associated with

Afrikaner ancestry (Reeves *et al.* 2004), is a single base pair substitution converting glutamic acid to a stop codon. To date, the mutation has not been submitted to the BIC database. E881X was detected in a female patient of Mixed Ancestry, contracting breast cancer at 40 years of age. This patient has three third degree relatives diagnosed with breast cancer. Kotze *et al.* (1995) showed in another study that a familial hypercholesterolemia (FH) Afrikaner founder mutation in the low-density lipoprotein receptor (*LDLR*) gene is also common in the Mixed Ancestry population. This suggests that the South African Caucasian and Mixed Ancestry populations may also share *BRCA1* and *BRCA2* variants. It is therefore plausible that the *BRCA1* Afrikaner mutation E881X was detected in a patient of Mixed Ancestry.

The third mutation, 5313delC, is in exon 20. The one base pair deletion causes a frameshift, introducing a stop codon in exon 21 at position 1764 and therefore deleting the C-terminal 5% of the protein. This deletion was identified in a female Caucasian patient diagnosed at age 52, reporting one second degree relative with breast cancer. This mutation is regarded as novel as it has not been reported in the BIC database.

5.1.2.3 Unclassified variants

Five variants that occur in coding regions or exons were detected, which have been reported to the BIC database as unclassified variants. These single nucleotide changes have therefore, to date, not been excluded as breast cancer susceptibility alleles. All of these are located in exon 11, which contains the binding domain for RAD51 (amino acids 158 to 1064), an interaction that is essential in the double-strand break repair function of the BRCA1 protein (Welcsh *et al.* 2000). One variant, T703T, is described in the database as an unclassified variant, but it does not result in an amino acid change and is therefore likely to be a benign polymorphism. This synonymous variant is also listed in the database as one of the 20 most common *BRCA1* mutations, which is another reason to consider T703T a benign polymorphism.

In N723D the change is from asparagine, an amide which is highly hydrophilic, to its acid, aspartate, which is also hydrophilic, but carries a negative charge and would therefore change the charge on the protein. This amino acid is, however, conserved in canine and murine species, suggesting functional significance of asparagine at this position. This is the only *BRCA1* unclassified variant detected in this study which is conserved across all three species (human, dog and mouse) (Table 4.2). Asparagine at

this position, however, does not seem to be part of a highly conserved motif and neither do any of the other novel unclassified variants.

In S1040N the change is from serine, a non-polar amino acid with an alcohol group, to the very hydrophilic asparagine, which is polar. This putative missense mutation was first described by Castilla *et al.* (1994) as a polymorphism. It was found not to segregate with the disease and was also detected in control chromosomes. In the report of Friedman *et al.* (1994), however, this alteration did segregate with the disease in the family in which it was identified and was absent in control chromosomes, therefore qualifying the variant as a missense mutation. Thus, the functional significance of the S1040N variant is questionable. The variant was only detected in one patient from our study population.

In S1140G the change is from serine to glycine. Both are small, non-polar aliphatic molecules. This variant has been detected in African American breast cancer cases and controls (Newman *et al.* 1998; Panguluri *et al.* 1999). In T1349M the change is from threonine, a non-polar amino acid with an alcohol group, to methionine, which contains a sulfur atom and is highly hydrophobic. The sulfide molecule present in methionine could cross-link with cysteine residues in other peptide chains and cause aggregations or clumping (Horton *et al.* 1996). The functional significance of both these variants is therefore not clear.

Three novel synonymous variants have been discovered. G677G and H1822H are postulated not to be pathogenic mutations, as they do not change the amino acid sequence. But, as mentioned before, they could have an impact on the mRNA level. H1822H cannot be detected with SSCP/HA, but has been detected in all samples sequenced (allele frequency of 0.1). It is therefore possible that this variant is a silent polymorphism that occurs at a relatively high frequency in the study population.

The third novel variant, K168T, has been identified in a previous study in our study population (Appendix C, Scholtz *et al.* 2003). This single base substitution cause an amino acid change, from lysine, a large highly hydrophilic, positively charged base to threonine, a small weakly polar amino acid with an alcohol group. Hydropathy, the relative hydrophobicity or hydrophilicity of each amino acid, is an important determinant of protein-chain folding. Lysine and threonine have very different properties and could therefore affect the three-dimensional structure as well as the binding properties of

the *BRCA1* protein. The variant, which is in exon 8 of the gene, falls just outside the N-terminal RING finger of the protein, amino acids 1-112 (Miki *et al.* 1994), which interacts with BARD1 and BAP1. This complex is subsequently involved in the ubiquitination of other proteins. Without functional, case-control or family studies, this variant should be considered an unclassified variant which could possibly have a functional role. K168T has been found in a female patient of Mixed Ancestry, who contracted breast cancer at the age of 68 years. She reported only one second degree relative with breast cancer. Unfortunately family members of this individual was not available for further investigation of this variant. However, 50 control individuals from an ethnically-matched population (Mixed Ancestry) were genotyped for this variant. The absence of the variant in this group supports a disease causing role for the K168T variant.

5.2 VARIANTS IDENTIFIED IN THE *BRCA2* GENE

5.2.1 Non-coding variants

In total, six variants were identified in non-coding regions of the *BRCA2* gene, of which two have been described in the BIC database (8035-15T>C and 9486-17T>C (Friedman *et al.* 1997)). Two of the novel variants occur in the 5'-untranslated region (5'UTR199T>G and 5'UTR212G>A), while one variant is 5' to exon 14 (in intron thirteen) (7236-27A>T) and another 5' to exon four (in intron three) (549-26C>T). Although the variant in intron three is not found in the database, it has been described by Wagner *et al.* (1999).

5.2.2 Coding variants

5.2.2.1 Benign polymorphisms

Eight known synonymous variants, H743H, V1269V and S2414S (Tavtigian *et al.* 1996), Q961Q, K1132K, L1521L and V2171V (Wagner *et al.* 1999) and L1356L were detected in the study population. Each of these only appeared in one, two or three patients, with the exception of K1132K and V1269V, which were found at a relatively high frequency (refer to Table 4.6 for genotype frequencies). The following variants are under the *BRCA2* top 20 synonymous mutations in the BIC database: H743H, S2414S, Q961Q and K1132K.

Six known amino acid altering polymorphisms were identified, all of which are single base substitutions. These are N289H (Couch *et al.* 1996a), N372H (according to the reference sequence, GenBank accession number U43746, the C allele is the wild-type but in the literature the polymorphism is commonly referred to as N372H, implying that 1342A is the wild-type genotype), N991D (Tavtigian *et al.* 1996), T1915M (Couch *et al.* 1996a), N1805S (Wagner *et al.* 1999) and V2138F. Of these variants, only two of the implicated amino acid residues (N289H and N1805S) are conserved in other mammalian species (dog and mouse) (see table 4.5).

The BRCA2 N372H non-conservative amino acid substitution falls within a region of BRCA2 (residues 290-453) that has been shown to interact with a histone acetyltransferase (P/CAF), needed for transcriptional activation of other genes (Fuks *et al.* 1998). Healey *et al.* (2000) found this polymorphism to be associated with an increased risk for developing breast cancer. Associations with this polymorphism were subsequently assessed in four other European population-based case-control series. Odds ratio (OR) estimates for individuals with the HH genotype were consistently elevated, ranging from 1.1 to 1.8 with the combined analysis yielding an OR of 1.3, explaining 2% of breast cancers occurring in those populations. Results from a large Australian population-based study (Spurdle *et al.* 2002) supported these findings. They found the HH genotype to be associated with a 1.4 to 1.5 fold increased risk for developing breast cancer. A comparison of genotypes among spontaneous abortions and live births suggested *in utero* selection against female fetuses with the HH genotype (Healey *et al*. 2000). Newborn females have an excess of heterozygotes and a deficit of homozygotes, whereas the opposite was observed in newborn males. The HH genotype was not absent in our female population, but occurred at a low frequency (4.35%). The study population demonstrated Hardy-Weinberg equilibrium, including (P=0.1013) and excluding (P=0.1723) the two male patients. Whether the variant has a direct causative role or is in linkage disequilibrium with a deleterious variant in *BRCA2* or another nearby gene, is not known yet (Goode *et al*. 2002).

5.2.2.2 Pathogenic mutations

Two deleterious mutations were detected, of which one is novel. The previously described mutation 8162delG (Gayther *et al.* 1997) is a frameshift mutation in exon 17. It introduces a stop codon at position 2647, therefore truncating the protein to ~77% of its full length (3418 amino acids). This would delete the nuclear localization signals (NLSs), rendering the protein cytoplasmic and ruling out

any interaction with the RAD51 complex (Spain *et al.* 1999). The mutation occurred in three patients, two Caucasian males and one female of Mixed Ancestry at a frequency of 6.1% in our study population. The male patients were diagnosed at age 53 years and 63 years respectively. The older male reported breast cancer in one first degree female relative. The female patient was diagnosed at age 46 years, but could not recall any family history of breast or ovarian cancer.

A novel mutation, 6677_6678insTA (can also be designated as 6675_6676dupTA), was identified in two female patients of Mixed Ancestry, one diagnosed at the age of 57 years and the other diagnosed at the age of 64 years. Neither reported a family history of breast cancer. This is another frameshift mutation, truncating the protein at amino acid residue 2167 (at ~63% of its full length). The truncation occurs in exon 11, which contains the important BRC repeats needed for RAD51 to be able to bind to the BRCA2 protein. This variant can therefore be classified as a disease causing mutation with relative certainty. This mutation is located 3' of the ovarian cancer cluster region (OCCR) in exon 11 (between nucleotides 3035 and 6629) (Gayther *et al.* 1997).

5.2.2.3 Unclassified variants

Three variants were found which have been reported in the BIC database as unclassified variants (M1149V, I3412V and K1285del). The M1149V variant was only identified in one patient (allele frequency of 0.01). Methionine and valine have similar characteristics, as both are large non-polar amino acids that are highly hydrophobic. However, methionine contains sulfur, which could create an illegal disulfide bond with another peptide. I3412V in exon 27 has first been reported by Teng *et al.* 1996 and is regarded as a rare sequence variant. Another group (Vehmanen *et al.* 1997) confirmed this as they could not detect the variant in control chromosomes. The functional status of this sequence change has not been elucidated to date. Isoleucine and valine have similar properties, as they are both aliphatic amino acids that are highly hydrophobic. Neither of the above mentioned substitutions occurs at conserved positions in canine and murine species. K1285del has initially been reported by Wagner *et al.* (1999). Although lysine is conserved in canine and murine species, it does not form part of a conserved motif (Table 4.5). K1285del and I3412V are in the BIC *BRCA2* top 20 lists for in frame deletions and missense mutations respectively.

Four variants were identified which are not listed in the BIC database. Of these, L775L and D3341D do not alter the amino acid sequence of the protein and will therefore not alter the structure of BRCA2. In addition to this, variants were detected which will change the primary structure of the protein. Two novel sequence variations were identified, N2447D and N1600del, of which the functional consequences are not known. The novel point mutation, N2447D, is caused by a A>G transition converting codon 2447 encoding the hydrophilic amide asparagine into aspartic acid or aspartate, its conjugated base (as the amino acid is found under most physiological conditions (Amino acids and the primary structure of proteins 1996)), which is also hydrophilic. The amino acids involved have similar properties and may therefore not have a major effect on the protein structure or function. Asparagine is also not conserved in either the dog or the mouse, which provides further reason to classify this variant as a benign polymorphism. N1600del is an in frame deletion, removing the residue aspartic acid from the peptide. Aspartic acid is neither conserved in canine, nor in murine species. Fifty control individuals from the Mixed Ancestry population were genotyped for this variant. The variant was not detected in any of these individuals. A functional consequence of this deletion can therefore not be excluded.

5.3 CONTRIBUTION OF *BRCA1* AND *BRCA2* MUTATIONS TO BREAST CANCER IN SOUTH AFRICAN FAMILIES

Due to the small sample size, this investigation should be considered a pilot study. A larger patient population need to be investigated to determine if the mutations identified, especially the novel mutations (*BRCA1* 5313delC and *BRCA2* 6677insTA), occur at an increased frequency in South African women with familial breast cancer. Apart from the E881X mutation, that has previously been shown to be a common mutation in the South African population (Reeves *et al.* 2004), the two mutations identified in the *BRCA2* gene, 8162delG and 6677insTA, had been identified in more than one patient each. Haplotype studies would indicate whether these patients had a common ancestor, or whether these are recurrent mutations. This was, however, not within the scope of this study.

Many unclassified variants have been identified in *BRCA1* and *BRCA2*, both known and novel. Functional implications for these variants cannot be ruled out, unless functional, family or case-control studies have been done. *BRCA1* exon 11 comprise 82.8% of the total coding area screened in this study and 92.9% (13/14) of the coding variants were detected in this exon. This does not include the variants

detected with melt-MADGE and not with SSCP/HA. The difference between the observed and expected number of variants is not significant if a homogenous distribution of mutations across the gene is expected. *BRCA2* exon 11 comprised 48.1% of the total coding area screened and 69.6% (16/23) of the coding variants were detected in this exon. The much higher frequency of exon 11 mutations in relation to the remainder of the gene could possibly be explained by the different screening methods used (SSCP/HA *versus* DS).

Deleterious *BRCA1* and *BRCA2* mutations have been detected in eight individuals. Only two of these individuals were diagnosed before the age of 50 years. None reported a family history of ovarian cancer or male breast cancer. Two patients had a one first or second degree relative with breast cancer; the other patients had a family history of breast cancer in more distant relatives. It is evident from these findings that early-onset breast cancer and a strong family history are not essential for identifying a *BRCA1* or *BRCA2* mutation. The accuracy and completeness of family history data, should however, be taken into account. A person may be unaware of relatives affected with cancer. In addition, small family sizes and premature deaths may limit the information obtained from a family history. In the case of breast cancer, cancer on the paternal side of the family usually involves more distant relatives than on the maternal side (due to the higher penetrance of *BRCA1* and *BRCA2* mutations in females relative to males) and thus may be more difficult to obtain. It is evident from population-based breast cancer studies that most women with a family history of breast cancer are not members of high-risk families, but instead have one, or perhaps two family members affected with breast cancer (Claus *et al.* 1998). Therefore, the extent to which a positive family history of breast cancer remains a factor in the prediction of breast cancer risk outside high-risk families remains an important issue. Our study population conforms to the profile for women (and men) at moderate risk of developing breast cancer. After determining the contribution of *BRCA1* and *BRCA2* mutations to breast cancer in this group of patients, one can now assess the appropriateness of predictive or diagnostic DNA testing in the clinical setting.

It has been shown in larger studies that *BRCA1* mutation carriers have a younger age of onset of breast cancer than *BRCA2* mutation carriers (Ford *et al.* 1998). More than 70% of our familial group have been diagnosed with late-onset (>50 years of age) breast cancer (see Table 3.1). This could explain the lower preponderance of *BRCA1 versus BRCA2* mutations in our study population, despite the higher contribution of *BRCA1* mutations in the general population (Krainer *et al*. 1997). Aretini *et al.* (2003)

suggests that less stringent eligibility criteria (moderate family history of breast cancer with little or no reported cases of ovarian cancer and later age at onset of breast cancer) would lead to a higher percentage of *BRCA2* mutations detected. The sensitivity of the screening method should also be taken into account. A large area of *BRCA2* (exon 11) was screened with a more sensitive technique (DS) than was the same area for *BRCA1* (exon 11, SSCP/HA).

In this study SSCP/HA has predominantly been used for mutation screening and SSCP is also the technique most often employed by other research groups for screening of the smaller *BRCA1* and *BRCA2* exons (refer to Table 2.3). The protein truncation test (PTT) is the most popular technique used to analyse the larger exons, namely *BRCA1* exon 11 and *BRCA2* exons ten and 11 (refer to Table 2.3). In addition to SSCP/HA, *BRCA1* exon 11 was screened with melt-MADGE. This technique proved to be less sensitive than SSCP/HA, as 60% of the exon 11 variants identified in our study group could not be detected with melt-MADGE analysis. This re-emphasises the fact that no method will be able to detect all the variants present in genes as large as *BRCA1* and *BRCA2*.

The variants detected may therefore not represent the full spectrum of mutations in the South African population, as the mutation detection technique used is not 100% sensitive. Large genomic rearrangements and mutations in regulatory regions have also not been screened for in this study. Since the Caucasian South Africans are of European ancestry and therefore also from The Netherlands, it is possible that the Dutch founder mutations, i.e. large deletions, will be present in the South African population. However, Reeves *et al.* (2004) did not detect any of these in their study population of South African breast/ovarian cancer families.

Deleterious *BRCA1* mutations have been identified in 6.3% and *BRCA2* mutations in 10.4% of patients from our study population. Only considering female patients, deleterious *BRCA2* mutations were only identified in 6.5% of the female study group. *BRCA1* mutations were identified in 5.9% female Caucasian patients. No *BRCA2* mutations have been found in female Caucasian patients, but *BRCA2* mutations have been identified in 100% of male breast cancer patients. *BRCA1* mutations were identified in 6.9% and *BRCA2* mutations in 10.3% of female Mixed Ancestry patients. Our populations of patients are mostly representative of cases who are of moderate risk for hereditary susceptibility, but who do not meet the stringent criteria of autosomal-dominant predisposition. The results of this study are in agreement with those from other populations, indicating that less than 20% of breast cancers that

occur in individuals with a modest risk for developing breast cancer are due to *BRCA1* and *BRCA2* mutations (refer to Table 2.3).

5.4 THE *CHEK2**1100DELC VARIANT AND THE SOUTH AFRICAN POPULATION

Although the *CHEK2**1100delC mutation has a stronger association with familial breast cancer, it has been reported to contribute to breast cancer risk in patients with sporadic disease (The CHEK2 Breast Cancer Case-Control Consortium 2004). We screened both familial (n=48) and sporadic (n=53) breast cancer patients, but the *CHEK2* mutation was not detected in our study population. Unfortunately we were not able to obtain a positive control for the mutation and no restriction enzyme sites are created or abolished by this variant. Two different SSCP/HA electrophoresis systems were therefore used to screen the patients. However, the detection technique is not 100% sensitive, and mutation positive individuals in the South African study population could have been missed. The sample size of 101 individuals was also relatively small, considering that the mutation have only been reported at a frequency of 2-6% in other populations (Meijers-Heijboer and The CHEK2-Breast Cancer Consortium 2002; Vahteristo *et al.* 2002; Walsh *et al.* 2002). The *CHEK2**1100delC mutation can therefore not be excluded as a low-penetrance breast cancer susceptibility allele in the two population groups screened.

6. CONCLUSION

Much of what is known about the frequency and distribution of germline *BRCA1* and *BRCA2* mutations derives from studies in those rare families that have large numbers of affected women and many living family members available for sampling. While detailed analysis of these mapping families has proven invaluable in studies aimed at determining the initial mutation spectrum, additional studies are now needed to elucidate the role of germline *BRCA* mutations outside the selected venue of high-risk families. Individuals with a modest breast cancer family history present the biggest challenge for the development of testing strategies in women from the general population. This will be further complicated by the intricate array of functions of the BRCA proteins and the uncertainty regarding most rare missense mutations and unclassified variants.

Overall, mutations in *BRCA1* and *BRCA2* are uncommon in the general population and account for a small proportion of all cases of hereditary breast cancer. This is evident from the results we obtained for our group of breast cancer patients with a moderate family history of the disease. Unselected screening for mutations is not feasible. It is therefore important to identify patients who have a high pre-test probability of carrying a mutation to justify genetic testing. Initial reports of breast cancer risk were overestimates since they depended in part on results obtained from high-risk families suitable for linkage analysis. Most of these studies have focused on women who were deliberately selected because they were members of large multiple member families with breast and ovarian cancer. Such women represent only a fraction of the spectrum of patients who seek advice in the clinical setting. Population specific risk estimates will aid in the selection and screening process. Our study is different in that we have characterized the *BRCA1* and *BRCA2* mutation spectrum in a population group unique to South Africa, namely the Mixed Ancestry population. In addition, we screened patients of European descent. None of the published European founder mutations have been detected in these patients (or the patients from Mixed Ancestry), suggesting a unique mutation spectrum for the people of South Africa. This observation emphasizes that we cannot only rely on data obtained from studies done in European, American or even British populations, but should conduct our own investigations using South African families as our study populations.

Two deleterious *BRCA2* mutations, 8162delG and 6677_6678insTA, have been identified in three and two patients respectively. A future prospect would be to establish if these mutations are recurrent mutations in the South African population or if the patients with these alleles have a common ancestor. Furthermore, the potential functional significance of both the known and novel *BRCA1* and *BRCA2* unclassified variants has to be assessed by screening control chromosomes. In addition to this, the *CHEK2**1100delC needs to be screened for in a larger group of breast cancer patients. Although this mutation was not detected in this study, it cannot be excluded as a low-penetrance breast cancer susceptibility allele, as the mutation has been identified in various populations and other research groups have established its functional significance.

The first recorded case of hereditary breast cancer dates back to 1757; a 19-year-old nun believed that "her blood was corrupted by a cancerous ferment natural to her family" (Le Dran 1757, as cited by Eisinger *et al.* 1998). Centuries later, the hereditary component have been calculated relatively accurately even before the identification of the *BRCA1* and *BRCA2* genes in the 1990s. It is, however, well recognized that there exists residual heritable risk that cannot be explained by mutations in these highly penetrant genes. The literature is currently mushrooming with small studies suggesting low penetrance gene effects that are then subsequently discounted by follow-up studies. An increasing number of studies are investigating the interaction between probable low penetrance alleles and environmental risk factors. These studies are vital since breast cancer is a complex disease, with a genetic and an environmental component. It is therefore comprehensible that, despite much research directed at understanding, preventing and treating breast cancer, it persists as a major health burden. To a certain extend, we can control and manipulate our environment, but we cannot change the genes and the genetic predisposition to disease that we have inherited. The challenge remains to identify and characterize our genetic inheritance to be able to ultimately implement cancer intervention programs.

"But remember throughout, that no external cause is efficient without a predisposition of the body itself. Otherwise, external causes which affect one would affect all." Galen, 200 A.D.

7. REFERENCES

7.1 GENERAL REFERENCES

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APPENDIX A

Patient consent form

SUBJECT INFORMATION AND CONSENT FORM

TITLE OF PROJECT:
The genetics of breast cancer

DECLARATION BY OR ON BEHALF OF PARTICIPANT

I, THE UNDERSIGNED, ……..…….. *(*name*)*

[ID No: ……..……] the participant/*in my capacity as ……...……. of

the participant [ID No: ……..……...] of …….

……...…… *(*address*).*

A. I declare that:

1. I/*The patient was invited to participate in the above mentioned research project which is being undertaken by the Department of Genetics, University of Stellenbosch, in collaboration with the Division of Human Genetics, University of Stellenbosch.

2. It has been explained to me that

2.1 the project is being undertaken to investigate the role of BRCA1 and BRCA2 mutations as a cause of cancer in the South African population.

2.2 if I/*The patient participate(s) in the project, only 10 ml (2 teaspoons) of blood will be collected from the arm. DNA and RNA will be extracted from the blood and used in genetic tests.

2.3 a blood sample will be required from both affected and unaffected family members. The project should not take more than 2 years. One blood sample per patient should be sufficient.

2.4 The research may provide important insights to our understanding of cancer. DNA diagnosis of breast cancer will assist in the overall management and counseling of familial cases and may contribute to our understanding of the role of BRCA1 and 2 in isolated cases of cancer.

3. I/*The patient have/has been warned that the drawing of blood may result in slight discomfort, which can be coupled with bleeding where the needle pierces the skin.

4. it has been explained to me/*the patient that participation in this project will result in the broadening of medical knowledge. The development of an accurate diagnostic DNA test for the condition(s) might lead to early detection and better counseling of affected individuals.

5. I/*The patient have/has been informed that all information collected will be treated confidentially. The results will be used for publication in scientific journals/theses, without revealing the identity of any individual.

6. I/*The patient may, during or on completion of the project, request the results of the tests without any conditions attached thereto, since the results could be advantageous to me/*the patient and my/*his or her family. Genetic counseling will be provided with this information.

7. I/*The patient have/has been told that participation is voluntary and that I/*the patient may refuse to participate in this project and that I/*the patient may also at any time withdraw my/*his or her participation from the project. Refusal or withdrawal from the project will in no way affect my/*his or her present or future treatment at this institution. I/*the patient also understand that the researcher may withdraw me/*him or her from the project if he/*she considers it in my/*the patient's best interest.

8. the information above has been explained to me/the patient by .....(name) in *English/Afrikaans/Xhosa, ...(other), and that I/*the patient am/is fully conversant in this language. I/*the patient was given the opportunity to ask questions which were answered to my/*the patient's satisfaction.

9. I/*the patient was not pressurized to participate in this project and I/*the patient know that I/*the patient may at any time withdraw from this project without penalization.

10. participation in this project will not result in unnecessary expenses for me/*the patient.

B. **I/*the patient voluntarily agree(s) to** participate in the above mentioned project

SIGNED/AFFIRMED AT…..... ON …..... 20.....

....
Signature or right thumb print of patient/representative of patient — Witness

DECLARATION BY OR ON BEHALF OF THE RESEARCHER

I, ...……..(name) declare that I

1. have explained the information in this document to the patient, ...……(name) and/or his/*her representative.
2. requested *him/her/them to ask questions where anything was unclear.
3. this conversation was conducted in *English/Afrikaans/Xhosa, ... (other) *and no translator was used/that this conversation was translated into ....by Dr/Mr/Ms .....(name)

SIGNED AT……..... ON .....20.....

....
Researcher/Research representative — Witness

DECLARATION OF TRANSLATOR

I, ...……..(name) declare that I

1. translated the contents of this document from *English/Afrikaans/Xhosa/..... (other) into ..... to the patient/*representative of the patient as well as the questions put to .....(name of researcher) by the patient/*patient's representative and translated this person's answers.

2. information translated by me is a factual account of what was conveyed to me

   SIGNED AT ..... ON ..... 20.....

   ....
   Translator's signature Witness

---

* Delete where not applicable

IMPORTANT INFORMATION

Dear Patient/Representative of the patient

Thank you for participating in this project. If at any time during the course of the project

1. you require further information regarding the project, or

3. if any of the following occurs, please contact **Prof Louise Warnich** at telephone number: **(021) 8085888**

   - a participant changes address and/ or telephone number.

APPENDIX B

Patient questionnaire form: English

Study Group Number MA ☐☐☐☐☐ Control Group Number ☐☐☐☐☐

Date of Interview: ☐☐☐☐/☐☐/☐☐ Interviewer:…………………………………….

Diagnosis (clinical): …………………………………….……… Stage: …………………..…….…

Breast Cancer Questionnaire for Patients

Personal Information

Name: ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Date of Birth: ☐☐☐☐/☐☐/☐☐ Age: ☐☐☐ Sex: M ☐ F ☐

Race: ☐Coloured ☐ White ☐Black ☐Indian ☐Other (please specify) .....…...........……………...

Ethinicity: ………………………………………………………………………………………..

Place of Birth: ……………………………………………………………………………………...

Disease States

Do you suffer from any other disease? ☐Yes ☐No If yes, indicate disease ……................…..………..

When were you diagnosed with breast cancer? (year/month/day) ☐☐☐☐/☐☐/☐☐

How old were you when you were diagnosed? ☐☐☐

Do you have unilateral or bilateral tumours? ……………………………………….………………….

If contralateral breast cancer has been diagnosed:

Date of diagnosis (year/month/day) ☐☐☐☐/☐☐/☐☐ Age at diagnosis ☐☐☐

Have you received treatment for your breast cancer? ☐Yes ☐No

	Name of drug	Response	Negative side effects
1			
2			
3			
4			
5			

Do you suffer from ovarian cancer? ☐Yes ☐No

Has any blood relative had cancer? ☐Yes ☐No

	Relative 1	Relative 2	Relative 3	Relative 4
Relationship (eg aunt, sister)				
Type of cancer				
Age at diagnosis				
If breast cancer, was it unilateral or bilateral				
Age at diagnosis of each				
Relative's current age				
Deceased from the cancer?				
Age of death				

Family Tree

Risk Factors

Menarche, pregnancy and breast feeding

How old were you at menarche? ☐☐☐years ☐☐months

Have you ever been pregnant? ☐Yes ☐No

How old were you when you first fell pregnant? ☐☐☐years ☐☐months

How old were you when you had your first live birth? ☐☐☐years ☐☐months

How many times have you been pregnant? ☐☐☐

How many

Live births	Miscarriages	Still births	Abortions

How long did you breast feed your children (average)? ☐☐☐years ☐☐months ☐☐days

Contraceptive Use

Have you ever taken contraceptives? ☐Yes ☐No

Please indicate the name of the contraceptives. ……..……..

……...…..……

How old were you when you started taking contraceptives? ☐☐☐years ☐☐months

How old were you when you last used contraceptives? ☐☐☐years ☐☐months

How long in total did you take contraceptives? ☐☐☐years ☐☐months

Why did you stop taking them? ……..……...

……

Hormone Replacement Therapy and Menopause

Have you been through menopause? ☐Yes ☐No

If yes, how old were you when you experienced it? ☐☐☐years ☐☐months

Have you been on hormone replacement therapy (HRT)? ☐Yes ☐No

What kind of HRT?

☐Oestrogen alone ☐Progesterone alone ☐Combination: ☐sequence ☐non-sequenced

Name of drug: ……..

How long did you receive HRT? ☐☐☐years ☐☐months

Other

Have you had a hysterectomy? ☐Yes ☐No At what age did you have it? ☐☐☐years

Were your ovaries left in? ☐Yes ☐No ☐Unsure

Have you previously had breast irradiation? ☐Yes ☐No

Do you take any other drugs/medication on a regular basis? ☐Yes ☐No If yes, please specify.

……..

Do you donate blood on a regular basis? ☐Yes ☐No

Do you take vitamin/iron supplements on a regular basis? ☐Yes ☐No If yes, please specify.

……..

Smoking

Do/did you smoke? ☐Yes ☐No

Age started? ☐☐☐years Age stopped? ☐☐☐years

Average number smoked per day: ☐☐☐

What did you smoke? ☐Cigarettes ☐Pipe ☐Hand-rolled cigarettes

☐Other (please specify) ……

How long did you smoke regularly? ☐☐☐years ☐☐months

Number of pack years smoked: ☐☐☐

Side-stream smoke

Did anyone smoke in your house/room? ☐Yes ☐No

Do people smoke around you in your workplace? ☐Yes ☐No

Have you ever lived or worked in a smoky environment? ☐Yes ☐No

When? (year/month/day) ☐☐☐☐/☐☐/☐☐

Alcohol

Do/did you drink alcohol? ☐Yes ☐No

What did you drink? ☐Beer ☐Wine ☐Spirits

☐Other (please specify) ……

How long did you drink regularly? ☐☐☐years ☐☐months

On average, how many drinks would you have per sitting? ☐☐☐

Patient questionnaire form: Afrikaans

Studiegroep Nommer MA ☐☐☐☐☐ Kontrolegroep Nommer ☐☐☐☐☐

Onderhoud Datum: ☐☐☐☐/☐☐/☐☐ Onderhoudvoerder:…….

Diagnose (klinies): ……..…… Stadium: ……...……..…

Borskanker Vraelys vir Pasiënte

Persoonlike Inligting

Naam: ☐☐

Geboortedatum: ☐☐☐☐/☐☐/☐☐ Ouderdom: ☐☐☐ Geslag: M ☐ V ☐

Ras: ☐ Kleurling ☐ Blank ☐ Swart ☐ Indiër

☐ Ander (spesifiseer asb.) .....….....…….……..

Etniese Agtergrond: ….……..

Geboorteplek: ……...

Siekte Status

Lei u aan enige ander siekte(s)? ☐Ja ☐Nee Indien ja, spesifiseer asb …….…….....….……..

Wanner is u gediagnoseer met borskanker? (jaar/maand/dag) ☐☐☐☐/☐☐/☐☐

Op watter ouderdom is u gediagnoseer? ☐☐☐

Het u unilaterale of bilaterale gewasse? ……..…….

Indien kontralaterale borskanker gediagnoseer is:

Datum van diagnose (jaar/maand/dag) ☐☐☐☐/☐☐/☐☐ Ouderdom met diagnose ☐☐☐ jaar

Het u behandeling ontvang vir borskanker? ☐Ja ☐Nee

	Naam van behandeling	Reaksie	Newe effekte
1			
2			
3			
4			
5			

Het u ovariale kanker? ☐Ja ☐Nee

Het enige bloedverwante familie kanker gehad? ☐Ja ☐Nee

	Familielid 1	Familielid 2	Familielid 3	Familielid 4
Verwantskap (bv. tante, suster)				
Tipe kanker				
Ouderdom met diagnose				
Indien borskanker, was dit unilateraal of bilateraal?				
Ouderdom met diagnose				
Familielid se huidige ouderdom				
Oorlede aan kanker?				
Ouderdom met afsterwe				

Familie stamboom

Risiko Faktore

Menarche, swangerskap en borsvoeding

Ouderdom met menarche? ☐☐☐jaar ☐☐maande

Was u ooit swanger? ☐Ja ☐Nee

Wat was u ouderdom met u eerste swangerskap? ☐☐☐jaar ☐☐maande

Op watter ouderdom het u u eerste lewende geboorte gehad? ☐☐☐jaar ☐☐maande

Hoeveel keer was u swanger? ☐☐☐

Hoeveel

Lewende geboortes	Miskrame	Stil geboortes	Aborsies

Vir hoe lank het u u kinders geborsvoed (gemiddeld)? ☐☐☐jaar ☐☐maande ☐☐dae

Voorbehoedmiddel gebruik

Het u ooit voorbehoedmiddels gebruik? ☐Ja ☐Nee

Dui asb. die naam van die middel(s) aan: ……..……..

……...…..……

Op watter ouderdom het u voorbehoedmiddels begin gebruik? ☐☐☐jaar ☐☐maande

Op watter ouderdom het u laas voorbehoedmiddels gebruik? ☐☐☐jaar ☐☐maande

Vir hoe lank in totaal het u voorbehoedmiddels gebruik? ☐☐☐jaar ☐☐maande

Waarom het u opgehou om dit te gebruik? …….……...

……

Hormoonvervangingsterapie en Menopause

Het u reeds menopause ondergaan? ☐Ja ☐Nee

Indien ja, op watter ouderdom het u dit ondervind? ☐☐☐jaar ☐☐maande

Was / is u op hormoonvervangingsterapie (HVT)? ☐Ja ☐Nee

Watter soort HVT?

☐Estrogeen alleen ☐Progesteroon alleen ☐Kombinasie: ☐sekwens ☐nie-sekwens

Naam van middel: ……..

Vir hoe lank het u HVT ontvang? ☐☐☐jaar ☐☐maande

Ander

Het u ‘n histerektomie gehad? ☐Ja ☐Nee Op watter ouderdom? ☐☐☐jaar

Is u ovaria ook verwyder? ☐Ja ☐Nee ☐Onserker

Het u voorheed bestraling van die bors gehad? ☐Ja ☐Nee

Neem u enige ander medikasie op ‘n gereelde basis? ☐Ja ☐Nee Indien ja, spesifiseer asb.

…….

Skenk u gereeld bloed? ☐Ja ☐Nee

Neem u gereeld vitamien / minerale aanvullings? ☐Ja ☐Nee Indien ja, spesifiseer asb.

…….

Rook

Rook of het u gerook? ☐Ja ☐Nee

Ouderdom waarop begin? ☐☐☐jaar Ouderdom opgehou? ☐☐☐jaar

Gemiddelde aantal gerook per dag: ☐☐☐

Wat het u gerook? ☐Sigarette ☐Pyp ☐Handgerolde sigarette

☐Ander (spesifiseer asb.) ……

Vir hoe lank het u gereeld gerook? ☐☐☐jaar ☐☐maande

Aantal jare gerook x pakkies per dag: ☐☐☐

Sekondêre rook

Rook iemand in u huis / kamer? ☐Ja ☐Nee

Rook mense rondom u by die werk? ☐Ja ☐Nee

Het u ooit gewoon of gewerk in ‘n rokerige omgewing? ☐Ja ☐Nee

Wanneer? (jaar/maand/dag) ☐☐☐☐/☐☐/☐☐

Alkohol

Drink of het u alkohol gedrink? ☐Ja ☐Nee

Wat het u gedrink? ☐Bier ☐Wyn ☐Spiritualeë

☐Ander (spesifiseer asb.) ……

Vir hoe lank het u gereeld gedrink? ☐☐☐jaar ☐☐maande

Gemiddeld hoeveel drankies het u per geleentheid gehad? ☐☐☐

APPENDIX C

Conferences at which research was presented

Results have been presented at the following national conferences. The presenting author's name is underlined.

ORAL PRESENTATIONS

10th BIENNIAL CONGRESS OF THE SOUTHERN AFRICAN SOCIETY OF HUMAN GENETICS: Durban, South Africa, May 2003. Scholtz CL, Agenbag GM, Du Plessis L, Thiart R, De Villiers JNP, Bouwens CSH, Warnich L, Apffelstaedt J, Kotze MJ. Molecular-genetic analysis of South African patients with breast cancer: Identification of novel mutations in the *BRCA1* gene.

POSTER PRESENTATIONS

18th CONGRESS OF THE SOUTH AFRICAN GENETICS SOCIETY: Stellenbosch, South Africa, April 2004. Agenbag GM, Scholtz CL, Apffelstaedt J, Warnich L, Kotze MJ. Molecular-genetic analysis of the *BRCA1* gene in South African breast cancer patients.

11th BIENNIAL CONGRESS OF THE SOUTHERN AFRICAN SOCIETY OF HUMAN GENETICS: Muldersdrift, South Africa, March 2005. Agenbag GM, Kotze MJ, Apffelstaedt J, Warnich L. Molecular-genetic analysis of the *BRCA2* gene in South African breast cancer patients.